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CLINICAL AND EXPERIMENTAL

STUDIES ON A POLYSACCHARIDE FROM THE TUBERCLE BACILLUS*

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REFERENCES to the role played by polysaccharide substances in pathologic and nonpathologic processes are numerous and date back some 40 years, when Emerling,[1] and Schardinger,[2] reported the isolation of a nonnitrogenous polysaccharide from *Bacillus lactis aerogenes*. Our more recent knowledge of the chemical and immunologic specificity of polysaccharide materials, especially from the pneumococcus and tubercle bacillus, has been derived in a large measure from the work of Landsteiner, Heidleberger, Avery, Goebel, Sabin, and their associates. Other workers have contributed information concerning this subject.

In view of the above work and since no definite role has been assigned to the polysaccharide fraction in the pathogenesis of tuberculosis, it seemed worth while to attempt the following type of experiment.

MATERIAL AND METHODS

The fraction was isolated from the aqueous extract (2000 c.c.) of approximately 4 kilograms of moist germ mass which had been grown on Dorset's synthetic medium. The bacillary mass was mechanically agitated in its aqueous liquor for one month previous to the process of extraction. The fraction when ready for clinical testing was free from organic nitrogen, as was determined by the biuret, ninhydrin, and the sodium fusion (Lassaigne) tests. The material gave a strong Molisch reaction, indicative of carbohydrate substances; it does not reduce before hydrolysis but reduces alkaline copper reagents after hydrolysis in 1 N Hcl. Tests for combined pentoses were positive. The optical rotation (observed) was 58 D. The naphthoresorcinol test for glycuronic acid was

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negative. The fraction was completely soluble in water at ordinary room temperature. Further chemical investigation of the carbohydrate material is now under way.

TOXICITY

The tests for toxicity were carried out in tuberculous and nontuberculous guinea pigs. Temperatures were taken in each case before any injections were made. Twenty milligrams in 1 c.c. of normal saline solution given intraperitoneally was found to be the lethal dose for animals with generalized tuberculosis. Death followed within five to eight hours after administration of the lethal dose. Normal saline solutions were injected into control animals. The carbohydrate material produced a rise of 3 to 5 degrees in the tuberculous animals and from 1 to 2 degrees in nontuberculous animals. The temperatures in the infected animals continued to fall to 95° F. at the time of death. In the nontuberculous animals the temperatures did not fall to more than 2 degrees below the temperatures taken before injection; none of the nontuberculous animals succumbed to the dosage used. No rise in temperature was observed in the control animals given 1 c.c. of the saline solution alone. It was also noted during post-mortem examination of the tuberculous animals that those with far-advanced disease succumbed sooner than those with less or minimal amounts. Death in the tuberculous animal followed three to eight hours after injection, depending upon the extent of disease present.

CLINICAL

After repeated animal experiments of the above character, one of the writers (G. V. K.) and 14 members of the hospital staff received intracutaneous injections of 0.01 mg. in 0.1 c.c. amounts of the material made up in normal saline solution. Saline controls were used by similar route in the opposite arm, the saline being the same solution used for dilution of the carbohydrate material. Five of the latter group were arrested cases of pulmonary tuberculosis; the remainder had no history of active disease. The results of the skin tests in this group are listed in Table I.

It has been noted during injections of this carbohydrate fraction that when the material is administered subcutaneously, no reaction is seen in the skin whether in tubercular or nontubercular individuals. It has also been observed that in each instance when a positive skin reaction is elicited there occurs a concomitant change in the differential blood picture, i.e., a rise in lymphocytes with a fall in the monocytes and a shift to the right in the Schilling index. The latter change has, in the majority of cases, been correlated with the degree of skin reactivity. For example, the differential blood count of an individual (tubercular or nontubercular) with a ± or a + skin reaction (immediate reaction) has never reflected any noteworthy change following intracutaneous injections of this polysaccharide fraction. As a matter of note, the immediate type of reaction, although in many instances has been correlated with the clinical improvement of the patient, has nevertheless not been constant enough to permit any definite conclusions at this time regarding its clinical significance.

An unusually severe reaction to the intracutaneous injection was seen in subject F. B. An area of erythema appeared within 2 hours following the injection; this gradually became intensely indurated and at its peak measured 8 cm. in diameter. One of the axillary nodes became enlarged and tender. The reaction did not subside until 48 hours later and it was not accompanied by any febrile reaction or local necrosis. This individual gave no past history of active disease.

In each case, total white, red, and differential blood counts were made one hour before injections were given, and counts were again made 8 hours following the injection. In the absence of any skin reactivity whatsoever, no changes in the blood picture have ever been observed. The time of appearance of a positive test in a case of minimal disease, employing the usual dosage of .01 mg., is approximately 3 hours from the time of injection and is completely faded at the end of 20 hours. In the instance of far-advanced disease, however, the reaction is delayed, and if it appears at all, it will not do so until 10 to 12 hours following injection. In many far-advanced cases of this type the increment of dosage has had to be increased (i.e., 0.01 mg. to 0.1 mg.) in order to elicit a local reaction, which then might appear in 5 to 10 hours or longer. In such cases, the reaction has been seen to persist for from 72 to 124 hours.

TABLE I

SKIN REACTION IN ARRESTED AND NONARRESTED SUBJECTS

SUBJECT	SEX	AGE	AMOUNT	ROUTE	LOCAL REACTION	
					IMMEDIATE	DELAYED
A. C. ××	M	36	0.01 mg.	I.V.	+	++ (4 hrs.)
A. R. ××	M	33	0.01 mg.	I.V.	+	++ (3 hrs.)
W. R. ××	M	31	0.01 mg.	I.V.	+	++ (7 hrs.)
A. M. ××	M	44	0.01 mg.	I.V.	+	++ (4 hrs.)
W. K. ××	M	45	0.01 mg.	I.V.	+	++ (3 hrs.)
M. G.	F	23	0.01 mg.	I.V.	±	0
C. W.	M	30	0.01 mg.	I.V.	0	++ (2 hrs.)
P. C.	M	30	0.01 mg.	I.V.	±	0
G. M.	M	28	0.01 mg.	I.V.	+	0
E. R.	F	23	0.01 mg.	I.V.	0	++ (6 hrs.)
G. M.	F	29	0.01 mg.	I.V.	0	0
E. J.	F	31	0.01 mg.	I.V.	±	++ (4 hrs.)
M. B.	M	33	0.01 mg.	I.V.	0	0
F. B.	M	34	0.01 mg.	I.V.	0	++ (48 hrs.)
G. V. K.	M	38	0.01 mg.	I.V.	+	0 (15 hrs.)

+ Wheal with erythema and no induration 2 cm. or more; 5′ to 15′.
++ Wheal with erythema and induration 2 cm. or more; 2 to 10 hours' duration.
×× Arrested cases of at least 2 years' standing.
± Erythema less than one cm., with no induration.

In Table I there is noted a delayed reaction in three individuals with no past history of disease. We have not as yet been able to establish any correlation in such instances between the individual's past history or present status and the skin reaction. The discussion of the latter type of reaction will be considered under the serologic heading. On repetition of the same dose, the size and intensity of the skin reaction is seen to fade until a local reaction is no longer elicited. The phenomenon is dependent upon the intensity and duration of the initial reaction. As mentioned above, a local reaction is again elicited when the increment of dosage is increased.

For the sake of brevity, and because more detailed tables and data are presently being prepared, we wish to present a group of 67 patients with minimal and far-advanced disease in whom injections of the polysaccharides were given without hematologic or serologic study, in an endeavor to show some correlation between the local reaction and the extent of disease present in the individual case. In Table II is a summary of the patients thus studied:

TABLE II

LOCAL INTRACUTANEOUS REACTIONS IN MINIMAL AND FAR-ADVANCED DISEASE

NO. OF PATIENTS	STAGE OF DISEASE	RANGE OF DOSE	AV. TIME OF APPEARANCE OF LOCAL REACTION	AV. TIME OF DURATION OF LOCAL REACTION
22	Minimal	0.001 mg. to 0.01 mg.	38 hrs.	19.7 hrs.
45	Far Advanced	0.01 mg. to 0.3 mg.	10.8 hrs.	52.4 hrs.

Our observations in this group justify us in saying that (a) the time of appearance, (b) the intensity and size of the local reaction, and (c) the duration of the local reaction are to be definitely correlated with the extent of involvement in each individual case. This observation suggests some reciprocal relationship to the toxicity tests performed earlier in guinea pigs, when it was noted that animals with far-advanced disease succumbed sooner to the dosage used then did those with a minimal amount. A similar group of patients are now being studied with accompanying hematologic and serologic studies.

SEROLOGIC

During the course of the above injections, attempts were made to demonstrate any antibody response which might be elicited by injections of the carbohydrate substance. For this purpose, human subjects were divided into three groups: (1) tubercular patients who had received 12 weekly injections in doses ranging from 0.001 mg. to 0.3 mg., (2) tubercular patients who had received no injections at all, and (3) nontubercular individuals who likewise had received no injections of the carbohydrate substance. The sera of all three groups were tested for the presence of antibody before any injections were made. The technique used in determining the presence of antibodies is as follows: 5 c.c. of blood were drawn from the antecubital vein and placed in a glass tube and allowed to clot. The clot was later separated and the supernatant blood centrifuged at low speed. The sera were then heated at 55° C. for one hour. Dilutions in the sera of 1 to 10 were made in all instances.

The method of titration used was that of the simple ring test, in which the serum was overlayed with an 0.01 mg. per c.c. concentration of the carbohydrate fraction in saline. An equal number of saline-carbohydrate controls were also used. The observations made on the sera of the above subjects are given in Table III.

In Table III there are noted tubercular and nontubercular individuals as well as a convalescent patient having pleurisy with effusion, whose sera contained homologous antibodies for the carbohydrate fraction previous to any injection of

the latter. This phenomenon is altogether possible in tubercular individuals, since the carbohydrate fraction is a component part of the bacillary body. The instance of the convalescent pleurisy with effusion might also be explained on this basis. It becomes, however, more difficult to explain the presence of demonstrable antibodies in the serum of nontubercular subjects who have no past history of active disease, with only the possibility of an earlier childhood type of infection. The persistance of circulating antibodies for an extended period of time is possible since one cannot easily determine the status quo of a primary complex. Recent exposure to respiratory and other types of infection must also be considered. Since the above subjects are members of the sanatorium staff and have been in intimate contact with tuberculous patients for a period of time ranging from two to seven years, the presence of circulating antibodies in these individuals is understandable on this basis.

TABLE III

SERUM REACTIONS OF A VARIETY OF SUBJECTS TO THE CARBOHYDRATE FRACTION

	SUBJECT	CLINICAL STATUS*	NUMBER INJECTIONS	AMOUNT	SERUM BEFORE INJECTION†	SERUM AFTER INJECTION†	SALINE CONTROLS
Tubercular Non-treated	H. F.	Min.	0		0	0	0
	R. K.	F. A.	0		0	0	0
	M. F.	Min.	0		×	0	0
	W. C.	F. A.	0		0	0	0
	H. L.	Min.	0		0	0	0
Tubercular Treated	M. K.	F. A.	10	0.001 mg.–0.3 mg.	0	××	0
	H. S.	F. A.	10	0.001 mg.–0.3 mg.	0	×	0
	R. P.	F. A.	10	0.001 mg.–0.3 mg.	0	×	0
	M. C.	Min.	10	0.001 mg.–0.1 mg.	0	××	0
	W. D.	Min.	10	0.001 mg.–0.1 mg.	0	×××	0
Non-tubercular Nontreated	P. C.	Nontb.	0		0	0	0
	E. J.	Nontb.	0		0	0	0
	A. M.	Nontb.	0		×	0	0
	A. C.	Nontb.	0		×	0	0
	C. W.	Nontb.	0	0	0	0	0
Convalescent patient Pleurisy with effusion	R. S.	Nontb.	0	0	××	0	0

*Min. = minimal disease
F.A. = far-advanced disease
†× = slight ring at junction of fluids
×× = medium ring at junction of fluids
××× = heavy ring at junction of fluids

The type of antibodies elicited by intracutaneous injections of this material, from determinations made thus far, has been precipitin in nature only. The fact that the polysaccharide fraction used in the present experiment has repeatedly stimulated the homologous type of antibody might lead one to question how a hapten-like material may apparently act as a complete antigen. Investigations on polysaccharide substances have shown them to be haptens because of their failure to stimulate antibody formation, the latter being a distinction set forth by Landsteiner. More recent observations, however, have been recorded, and they suggest that certain of the more complex haptens may act as antigens under definite conditions. Thus, the polysaccharide fractions that have been separated from various types of pneumococci, when tested by

ordinary methods of inoculation into laboratory animals, have no antigenic action; on the other hand, when these haptens are injected into the skin of human subjects in minute doses, specific antibodies are formed (Francis and Tillet,[3] Finland and Sutliffe.[4] The animal and human experiments with the fraction used in this instance corroborates the observations of the above-mentioned workers; namely, that no antibody response has been demonstrated by any other route than the intracutaneous one.

Since the question of protein-nitrogen-contaminating polysaccharide fractions is constantly being raised, we felt it worth while to skin-test a group of 17 nontubercular individuals with the purified protein derivative (P.P.D.) and our polysaccharide fraction. The age range in this group was from 6 months to 49 years. The entire group was first tested with P.P.D. The first strength dose of 0.0002 mg. was administered on the volar aspect of the forearm. Two weeks after all reactions to the protein had subsided, the group was tested with 0.01 mg. of the polysaccharide fraction. The results of these tests are summarized in Table IV.

These cases are being presented for what they are worth; we feel, however, that the results suggest some difference in the nature of the two fractions used. The results could be discussed at length from several points of view; we are, however, reserving this discussion until a similar and larger group which is now being studied in greater detail is completed.

To further investigate the nature of these antibodies, protection experiments against what had previously been found to be the lethal dose in tubercular guinea pigs were carried out with the serum of the three types of subjects mentioned in Table V.

For this purpose tuberculous animals were used. All of the latter had been inoculated four weeks previously with 0.01 mg. strain H37 in the right groin and reacted with two and three plus skin reactions to 1 O. T. intracutaneously. The sera of all the patients used in this experiment were tested for the presence of antibodies previous to injection in the animals. In all the sera of the patients used, there were no demonstrable antibodies found, with the exception of the treated group. Since no quantitative methods were used in the titration of the above sera, we are unable to state definite titers of antibodies occurring in the sera used. One animal in the treated group succumbed to the lethal dose. This animal when sacrificed was found to have an extremely far-advanced disease. Likewise, when using the sera of the non-treated tubercular patient, one animal survived the lethal dose. This particular patient had previously shown definite clinical improvement.

This is in keeping with some earlier observations which were made with the serum of patients who had shown general clinical improvement with marked clearing in their x-ray report. The majority of these patients' sera reacted with the polysaccharide material in the dilutions and technique mentioned above. Since the carbohydrate substance with which we are here concerned is still in the process of chemical study, and since the serologic techniques here employed are crude, one cannot be dogmatic in the interpretation of the above results. For instance, the fact that the precipitin type of antibody was the

only type demonstrable does not rule out the possibility of other neutralizing agents which are not easily demonstrated in vitro and come under the heading of protective type of antibodies. More definite conclusions on this particular

TABLE IV

SKIN TESTS IN A NONTUBERCULAR GROUP

P. P. D.					POLYSACCHARIDE FRACTION	
CASE NO.	AGE AND SEX	DOSE AND ROUTE	LOCAL REACTION†	2 WEEKS LAPSING TIME	DOSE AND ROUTE	LOCAL REACTION
1	♂ 6 mos.	0.0002 mg. I. Q.*	+		0.01 mg. I. Q.*	−
2	♀ 6 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
3	♀ 16 yrs.	0.0002 mg. I. Q.	−		0.01 mg. I. Q.	+
4	♂ 17 yrs.	0.0002 mg. I. Q.	−		0.01 mg. I. Q.	+
5	♀ 19 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
6	♂ 20 yrs.	0.0002 mg. I. Q.	−		0.01 mg. I. Q.	+
7	♂ 24 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
8	♀ 25 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
9	♀ 25 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
10	♂ 30 yrs.	0.0002 mg. I. Q.	−		0.01 mg. I. Q.	+
11	♀ 23 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
12	♂ 6 yrs.	0.0002 mg. I. Q.	−		0.01 mg. I. Q.	−
13	♀ 31 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
14	♂ 36 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
15	♀ 49 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
16	♂ 30 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+
17	♂ 39 yrs.	0.0002 mg. I. Q.	+		0.01 mg. I. Q.	+

*Intracutaneously.

† + = Erythema and induration 2 cm. or more in diameter.

TABLE V

PROTECTION EXPERIMENTS IN TUBERCULAR GUINEA PIGS WITH TUBERCULAR-TREATED, TUBERCULAR-NONTREATED, AND NON-TUBERCULAR SUBJECTS' SERUM

ANIMAL	SERUM	AMOUNT AND ROUTE	TIME OF INJECTION	LETHAL DOSE AND ROUTE	TIME OF INJECTION	RESULTS	TIME
Buff	Treated Tb. +	2 c.c. I.V.*	9 A.M.	20 mg. in 2 c.c. I.P.†	10 A.M.	Killed	48 hrs.
Black	Treated Tb. +	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Killed	48 hrs.
Red and White	Treated Tb. +	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	16 hrs.
White	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Killed	48 hrs.
Brindle	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	12 hrs.
Brindle and Black	Non-Treated ++ Tb.	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	48 hrs.
Black and White	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	11 hrs.
Brown and White	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	15 hrs.
Brown and Buff	Non-Tb. +++	2 c.c. I.V.	9 A.M.	20 mg. in 2 c.c. I.P.	10 A.M.	Died	20 hrs.

+ Tubercular patients having received 12 weekly injections of the carbohydrate fraction in doses of 0.01 mg. to 0.1 mg.

++ Tubercular patients having received no injections.

+++ Nontubercular individuals.

*I.V. Intravenous.

†Intrapleural.

phase of the work will be possible only, when present chemical, animal, and human investigations are completed.

As was mentioned previously, there follows after each injection of this material (providing a positive skin reaction, delayed type) a change in the differential blood picture, characterized by a rise in lymphocytes and fall in monocytes with a concomitant shift to the right in the Schilling index. We have purposely not included tables or charts concerning these changes, because a larger group of tubercular and nontubercular subjects, along with animal experimentation, is presently going on in an attempt to establish a definite correlation between the subject's present disease status and (1) the hematologic, (2) the serologic, and (3) the skin reactions following intracutaneous injections of the carbohydrate material. It is nevertheless possible for us to state at this time that in instances where there has been an antibody response to injections of this fraction in tubercular individuals, such a response has always been preceded by a change in the lymphocytic cells as mentioned above. The latter observation is in keeping with the recent report of Ehrich and Harris.[5] The changes produced in the differential blood picture during the course of injections with this carbohydrate fraction varied greatly in their duration from subject to subject. Some blood changes were seen to persist only during the height of the local reaction; others continued for two to three days after the local reaction had subsided. We have not, as yet, made any correlation on this aspect of the problem other than to associate it with the extent of disease in the individual case.

DISCUSSION

In reviewing the literature pertaining to the leucocytic blood picture in tuberculosis, there is an unquestionable relationship between the lymphocytic-monocytic ratio and clinical status of the patient. From observations made on this subject, an increase in the number of lymphocytic cells and a corresponding fall in monocytes present a blood picture which in the large majority of instances has been closely related to the clinical improvement of the patient. From our own studies with the polysaccharide fraction we have found the afore-mentioned observations to hold true in almost all of our cases. While we do not have, as yet, any data on hand to explain the mechanism by which this polysaccharide fraction influences the lymphocytic tissue, or to assign any definite role to the lymphocyte in tuberculosis, we wish to quote the recent work of Ehrich and Harris.[5] Although the antigens used in their work (typhoid vaccine and sheep erythrocytes) are different from the one used in our own experiments, the mechanism of response in both instances is strikingly similar. They write in conclusion: "Antibodies began to appear in the efferent lymph 2 to 4 days after injections of the antigen and reached their highest titre after 6 days. This was preceded by a sharp rise in the output of lymphocytes through the efferent lymph, while in the lymph nodes there was lymphatic hyperplasia after preliminary infiltration of granulocytes and monocytes. The fact that the tissue response accompanying the formation of antibodies was chiefly a lymphocytic one points to the latter as a factor in the formation of antibodies."

In our own studies in human beings we have corroborated the latter observation, since a rise in lymphocytes has always preceded the demonstration of any antibodies. Our technique consists of weekly injections of the dosage found necessary to elicit skin reactivity: i.e., usually from 0.001 mg. to 0.1 mg., dependent upon the extent of disease in the individual patient. The primary response seen in the blood has always been a lymphocytic one. The earliest antibody appearance in any of our cases was after 12 weekly injections of doses ranging from 0.01 mg. to 0.2 mg. Our increment of dosage is guided by the persistence of the skin reaction; that is, the increment is increased only after cessation of skin reactivity to the present dose. Experiments have already been mentioned concerning the antibody response.

At present we have no experimental evidence of the role played in tuberculosis by either the antibody or the lymphocyte stimulated by injections of our polysaccharide fraction. Krause, in his essay "The Evolution of the Tubercle," writes, "In the elementary tubercle of ordinary experiment there exists besides the epithelioid cell, the lymphocyte, which in this location does not change its morphological character." Furthermore, the observations of Maximow[6] and Gerard[7] on tissue culture growths conclude that the lymphocyte is a transitional cell and enters into the mechanism of tissue repair.

While the above observations are interesting and although we have made our own notations along this line, we do not mean to place the entire responsibility of repair in tuberculosis upon the lymphocytic tissue or upon the antibodies which might arise therefrom; we do, however, feel that the work done thus far on lymphocytic tissue by various workers should not be entirely cast aside, until further experimental evidence has been presented.

CONCLUSIONS

1. A protein-free polysaccharide fraction has been isolated from aqueous extracts of the tubercle bacillus that is innocuous in the amounts used in tuberculous and nontuberculous human beings.

2. Hematologic studies of people receiving intracutaneous injections of this polysaccharide indicate a definite trend in the lymphocytic-monocytic ratio, characterized by an increase in lymphocytes and a decrease in monocytes, with an accompanying shift in the Schilling index to the mature type of neutrophilic cell.

3. Patients having received at least 12 weekly injections of this carbohydrate fraction develop antibodies of an homologous type.

4. The antibodies formed in tubercular patients as a result of the above injections are capable of protecting guinea pigs with generalized tuberculosis from succumbing to lethal doses of the polysacchride fraction.

5. No noteworthy change in the lymphocytic-monocytic ratio is observed unless there is an accompanying positive skin test of the type mentioned in Table I.

6. The sera of some tubercular patients who have not received injections of the carbohydrate material and who have shown definite clinical improvement will protect guinea pigs with generalized tuberculosis against lethal doses of the carbohydrate fraction.

7. The sera of nontubercular humans thus far observed will not protect animals against the lethal dose of the fraction, unless the individual has elicited a typical local reaction.

8. The appearance and duration of the local reaction is related to the extent of disease.

9. Demonstrable antibodies have always been preceded by a rise in the percentage of lymphocytes following injections of the polysaccharide fraction.

10. The skin reaction produced by the polysaccharide fraction used in the above experiments differs in (1) time of appearance, and (2) duration, from the protein fractions used in routine clinical testing.

11. In no instance during the course of the above injections has there been noted any febrile reactions or local necrosis in the amounts used.

12. A group of nontubercular individuals are presented; they were tested with the protein fraction P.P.D. and the polysaccharide fraction used in the present experiment.

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CHLOROPHYLL: AN EXPERIMENTAL STUDY OF ITS WATER-SOLUBLE DERIVATIVES*

IV. The Effect of Water-Soluble Chlorophyll Derivatives and Other Agents Upon the Growth of Fibroblasts in Tissue Culture

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THE problem of wound healing is one which has been intermittently the object of intensive clinical investigation over the years because of its obvious importance not only in ordinary civilian practice but especially in wartime. More recently, laboratory methods have been utilized more and more in the search for a satisfactory answer to the question. Tissue culture technique has been found to lend itself particularly well to the testing of various agents for their growth-stimulating effects. By this procedure standard cultures of cells can be grown under uniform conditions, very much as feeding experiments are made use of in studying and assaying vitamin requirements and content of foods. Similarly, single agents in varying concentrations can be added to the basic culture medium, and an exact quantitative measurement of their effect on cell growth determined.

Because of the almost regular response clinically of longstanding chronic ulcerative lesions to the application of chlorophyll with the formation of healthy granulation tissue and subsequent epithelization, as reported by Boehringer,[1] Buergi,[2] Gruskin,[3] and others, it was decided to study the effect of chlorophyll on the growth of fibroblasts in tissue culture to determine if possible whether this agent, as claimed, actually possessed any growth-stimulating properties. Various control experiments were carried out simultaneously, using other substances currently in use or under investigation in respect to the whole problem of wound healing. The preparation used in these studies consisted of medicinally purified water-soluble derivatives of chlorophyll,† in the main, a saponified metal complex, presumably sodium copper chlorophyllin. This we had found from experience to be more satisfactory than the oil-soluble derivatives with which apparently Boehringer and Buergi worked.

The following agents were employed in this study, all except the "ultrafiltrate" in aqueous solution:

1. Chlorophyll,‡ 0.2 per cent
2. Castilian malva§ infusion made by steeping 10 Gm. of

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†Wherever the term "chlorophyll" is used in these studies, the water-soluble derivatives are meant, the term "chlorophyll" being used solely for the sake of brevity.

‡The aqueous soluble chlorophyll preparation was generously supplied by the Rystan Company of New York, sole appointee of the Lakeland Foundation, Chicago, in accordance with the regulations of the Federal Food, Drug, and Cosmetic Act regarding experimental therapeutic products.

§The Castilian malva was generously supplied by the Upjohn Company of Kalamazoo, Mich., in accordance with the regulations of the Federal Food, Drug, and Cosmetic Act regarding experimental therapeutic products.

powdered leaves in 100 c.c. of distilled water for one hour, filtering and autoclaving.

3. Methionine (Merck) 0.01 per cent
4. Sulfanilamide (Merck) 1 per cent
5. Ascorbic acid 0.01 per cent (Merck)
6. Brewer's yeast extract (B complex) 0.025 per cent (Mead)
7. Simms' "ultrafiltrate"*

PROCEDURE

The technique employed is essentially that successfully used by us for the past ten or twelve years in tissue culture studies.[4] Especially designed thin 0.5 cm. Pyrex glass or aluminum rings with an inside diameter of 4 cm. are mounted sterilely on a cover glass by the use of paraffin. The medium is then added to this chamber, the tissue fragments fixed in position, and the chamber sealed by another sterile cover slip. This entire chamber is then placed within a sterile Petri dish and incubated in the usual manner. By this technique we have an easily accessible means of washing our cultures and replenishing the medium. It furthermore gives us a large, flat surface area for microscopic examination and photography of the culture and permits the use of a relatively large number of individual explants under identical conditions, a matter of considerable importance in the statistical evaluation of such studies as these, in which individual variation in the behavior of colonies is so prone to occur.

The basic medium used has been a Tyrode-plasma-tissue extract combination in the proportions of 4-4-2 parts respectively. The Tyrode solution is the usual standard salt mixture; the plasma has been obtained from chickens and heparinized in the proportion of 1 mg. to each 5 c.c. of plasma; as a matter of convenience maternal mouse spleen has been used for the tissue extract. After much preliminary and comparative study of tissues from various animals as well as human biopsy material as a source of fibroblasts, the embryonic mouse heart was selected as the test tissue. This possessed the advantage of uniformity for comparative purposes, and likewise was almost constantly available from our breeding colony.

The embryonic mouse heart was minced into small fragments 3 to 4 mm. in diameter and thoroughly washed twice in normal salt solution to remove any adherent tissue extract which might act as a growth stimulant. The fragments were planted in the medium, their identity duly recorded on the cover slip, and their measurement taken. Five to ten fragments were thus inoculated into each culture dish. Readings were made routinely each twenty-four hours, and the growth in millimeters from the edge of the original tissue mass was recorded.

Approximately five hundred tissue cultures were grown, averaging fifty to sixty for each set of experiments. Variations in the concentration of the drug or substance under investigation were made in an attempt to see whether there was any optimal concentration in any instance. In general, it may be said that there was very little difference in the rate of cell growth within wide

*Courtesy of Dr. H. H. Simms, Columbia University, College of Physicians and Surgeons, New York, N. Y.

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limits of such concentration. Too great an excess did seem to exert a definite inhibitory effect. This might best be explained perhaps on a physical rather than a chemical basis.

Certain technical difficulties were encountered from time to time. A small proportion of the cultures (5 per cent) failed to grow at all. These were simply disregarded in the computations, as the number of such nonviable colonies was about the same in the several groups. The chlorophyll exerted a definite and fairly quantitative inhibitory effect upon the plasma-clotting mechanism* which vitiated one entire set of cultures. It was found for this reason that concentrations of over 0.2 per cent chlorophyll could not be used successfully with this particular technique, but that the typical optimal growth response was obtained with this concentration.

The Castilian malva offered a special problem, as in the initial experiments the great majority of cultures became contaminated with a mold in spite of the fact that the infusion had been filtered through cotton and autoclaved before using. We had to resort to fractional sterilization to overcome this difficulty. With this exception, contamination was negligible, less than 1 per cent, and such cultures were discarded from the final computations.

Every effort has been taken to make these readings as objective and critical as possible, the measurements being checked by two observers for the most part and frequently without prior knowledge as to which agent was being used.

DISCUSSION

The results obtained in these experiments are summarized in Table I. It seems possible to draw certain definite conclusions from an analysis of these figures. They likewise provoke much of speculative interest. There seems to be little purpose in discussing in detail the individual experiments from which this report has been assembled. To those who are interested in the "toil, tears, and sweat" which are involved in such tissue culture studies, the original records are all available. To those whose interest lies primarily in the possible practical application of such studies to the clinical problems of wound repair, such individual protocols would have but little significance.

Nevertheless, there are certain aspects of these studies which might be discussed briefly. In the first place, it is well to emphasize the quantitative nature of these studies. Were it not for the relatively great numbers, their interpretation might very justly be open to criticism. In almost every group there have been individual colonies within a single culture which have temperamentally varied greatly in their rate of growth from their fellows, either lagging or stepping up their cell division to an excessive rate. It is hard to explain satisfactorily such behavior. We believe that the laggards probably represent tissues which have been unduly traumatized in handling; and that the more aggressive colonies retain some of the growth-stimulating factors normally present in injured tissues through surface adsorption or inadequate washing in saline before planting. Such an hypothesis is more or less substantiated by actually macerating cells before explantation, with resultant failure of growth

*As a side issue, this effect upon clotting was studied on several samples of blood, and it was found that clotting could be delayed or actually inhibited by the addition of chlorophyll in concentrations of 1:200 to 1:50 to blood samples as drawn hypodermically for clotting time. This might be further explored as a possible substitute for heparin or dicoumarin therapy in surgery.

on culture or the addition of supplemental tissue extract to a control culture with corresponding accelerated and more profuse growth.

It will be noted from an examination of the table that the substances studied fall into three groups insofar as their initial effect on cell growth in tissue culture is concerned. The growth of cultures to which either vitamin B or C was added did not appreciably differ from the control cultures. The addition of sulfanilamide definitely slowed down the rate of growth, and this effect persisted throughout the period of observation. In the third group we find that all four agents studied gave evidence of having a certain, comparable stimulating effect upon cell growth during the first twenty-four hours. Even in this group, however, the chlorophyll effect stands out as being significantly greater than that of any of the other three substances. It is of particular interest to note that this initial effect is sustained throughout the duration of the experiments in the case of the chlorophyll, whereas it drops off within forty-eight to seventy-two hours in those cultures to which the methionine, Castilian malva, and Simms' ultrafiltrate had been added, and the subsequent growth more or less parallels that of the control cultures. This same stimulating effect of chlorophyll upon fibroblastic proliferation in tissue culture has been observed by Dunham.*

TABLE I

AVERAGE GROWTH OF FIBROBLASTS EXPRESSED IN MILLIMETERS

	24 HRS.	48 HRS.	72 HRS.	96 HRS.	120 HRS.
Control	10.8	37.4	67.8	99.3	132.5
Vitamin B	11.3	44.1	70.2	98.7	129.8
Vitamin C (Ascorbic Acid)	10.6	38.6	59.4	91.3	123.7
Sulfanilamide	5.3	18.3	31.7	48.8	63.4
Ultrafiltrate	23.2	44.1	69.3	86.4	95.6
Methionine	22.8	23.2	72.5	87.5	130.0
Castilian Malva	22.7	40.5	63.5	85.8	126.4
Chlorophyll (Aqueous Solution)	27.4	54.3	88.9	120.4	148.6

These studies have likewise emphasized the necessity of replenishing the supplementary stimulating agent in the medium in order to maintain the rate of growth of the culture. The practical application of this observation to the clinical use of such preparations is self-apparent. The control culture grows at an average rate of approximately 31 mm. per twenty-four hours after an initial lag period of six to eighteen hours. The same picture is noted in those cultures to which vitamin B complex or ascorbic acid is added. There is the same relatively prolonged lag period, followed by a steady, continuous growth apparently uninfluenced by these agents. On the other hand, the sulfanilamide appears to maintain a continuous depressant effect over a period of at least one week, following a single dose added initially to the medium, unless the dilution is extremely high (1:5000). In the latter case no abnormality in the growth curve develops as compared to the control. An almost comparable retardation in the rate of growth occurs at whatever point during the first five days that the sulfa drug is added.

*Unpublished Data; Dunham, W. B., New York Post-Graduate Hospital, New York, N. Y.

In the case of the Castilian malva, the Simms' ultrafiltrate, and the methionine, the initial stepping up of the growth curve is followed by a retardation in the rate of growth of the culture, and subsequent replenishment of the medium with these agents produces only a slight and temporary additional stimulating effect.

With the addition of as little as 0.05 per cent of chlorophyll to the culture medium at the time of its inoculation, there is a sharp reduction in the lag period. Well-defined growth can be demonstrated regularly within six to eight hours, and not infrequently migration of cells occurs within two to four hours. This acceleration of fibroblastic proliferation tends to reach its peak within thirty-six to forty-eight hours, following which the growth curve begins to flatten out. By replenishing the chlorophyll every forty-eight hours, however, the initial 24-hour lead of nearly 40 per cent greater growth is maintained, with a step-like curve. If, however, the culture is not replenished with chlorophyll by the end of the forty-eight-hour period, then the curve of growth slows down so that by the end of five to ten days it, too, parallels the control culture.

The nature of this biologic response of the fibroblast to the presence of chlorophyll is most obscure. Whether it is the pigment molecule as a whole or some individual component part of it which is the effective agent remains undisclosed. Whether the chlorophyll acts directly or indirectly as a catalyst or through some photosynthetic mechanism is but one of the problems which this study provokes and which await a solution at some time in the future.

These tissue culture studies tend to support the clinical observations of Buergi, Gruskin,* and others as to the apparent growth-stimulating effect of chlorophyll upon fibroblasts. The clinical implications in regard to its possible therapeutic value in the repair of wounds are obvious. Even in ordinary operative procedures it might well have a place through the elimination or reduction of the usual lag period before repair begins. In chronic indolent lesions such as anemic (varicose) ulcers and other chronic infections, especially those associated with a reduced blood supply, the use of such water-soluble chlorophyll preparations would seem to be thoroughly justified. Again, in the older patient, whose healing processes normally are notoriously retarded, chlorophyll would seem to offer a possible step forward in the treatment of the multitudinous surface lesions with which he seems particularly to be afflicted.

SUMMARY

1. The behavior of fibroblasts in tissue culture subjected to the action of various agents including vitamin B complex, vitamin C, sulfanilamide, Castilian malva, methionine, Simms' serum ultrafiltrate, and an aqueous soluble chlorophyll preparation is reported.

2. Vitamin B complex and vitamin C cause no change in the rate of growth of fibroblasts under the conditions of these experiments.

3. Sulfanilamide in concentrations of more than 1:5000 show a definite retarding effect upon such cell growth.

4. Castilian malva, methionine, and Simms' ultrafiltrate cause a prompt but temporary increase in the rate of growth during the first twenty-four to forty-eight hours, followed by a leveling off of the curve to approximate the control.

*Loc. cit.

5. Chlorophyll in concentrations of 0.05 per cent to 0.5 per cent, when added to comparable tissue cultures of embryonic fibroblasts, causes an almost immediate growth response with elimination of the usual six- to eighteen-hour lag period.

6. This growth-stimulating effect can be maintained by replenishing the medium with chlorophyll every forty-eight hours.

7. A discussion of the possible clinical application of this biologic reaction to the problem of wound healing, both traumatic and thermal, is presented.

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TRAUMATIC AUTOGRAFT OF SPLENIC TISSUE IN THE BODY WALL*

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THERE have been a few reports in the literature of implants of splenic tissue over peritoneum and omentum as a result of traumatic rupture of the spleen. The usual sequence of events is: traumatic rupture of the spleen, splenectomy, and, often years later, observation of the abdominal cavity at operation or autopsy, at which time multiple small nodules of splenic tissue are found studded over the peritoneal surfaces and omentum. At least 82 such transplants in one patient are mentioned by Shaw and Shafi,[11] about 75 in a case of Hamrick and Bush,[5] and 200 to 300 nodules in a case described by Lee.[7]. The small implants vary from microscopic size to as large as 2 cm. in diameter and are usually sessile growths, but may be pedunculated.

Most of the implants which have been described are superficial peritoneal masses, but in the case of Shaw and Shafi, one microscopic implant was noted in the wall of the gall bladder, one in the upper portion of the left ureter (a left nephrectomy appears to have been performed on the patient at the time of the splenectomy), and one was imbedded in the liver. It is presumed that this last nodule was a graft which developed in a laceration of the liver which occurred at the time of the original injury. One macroscopic pleural implant was also noted on the lateral aspect of the centrum of the eighth dorsal vertebra. The authors did not demonstrate a scar in the diaphragm, but believed that there must have been a small tear to account for this implant.

That this condition is not often seen is probably due to a combination of factors. Especially important is the infrequent survival of persons who suffer traumatic rupture of spleen (as mentioned by Hamrick and Bush[5] and the probability that relatively few of those who do survive the injury and splenectomy ever come to subsequent operation or autopsy so that the peritoneal cavity can be examined.

From consideration of experimental work, as well as case reports, it seems probable that splenic implants may be the rule rather than the exception in cases of traumatic rupture of the spleen. Several factors may influence the likelihood of implant, however. It is suggested by Hamrick and Bush that youth may be an important factor. They note that the cases where data are available have suffered the original trauma at an average age of slightly more than nine years.

It is possible that youthfulness of splenic tissue is an important factor in viability of this tissue for autografts, but it should be kept in mind that traumatic, subcutaneous rupture of normal spleen occurs most frequently in childhood,[12] and this may account in part for the greater frequency of the phenomenon

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in children. It is noted in different series that incidence of splenic rupture varies from 30 to 43.8 per cent for the age group 0 to 10 years,[12] In animals and in man, the spleen tends to become somewhat atrophic in old age, and this may have some effect on the probability of implant in older age groups. It has been observed in rabbits that autografts of spleen are, on the whole, more successful in young animals.[8]

Other factors which may influence the likelihood of implantation are by no means clear. In their review, Jarcho and Andersen[6] reported two cases, discussed twelve other cases, and examined certain of the older theories suggested to account for the presence of many splenic nodules over the peritoneum after recovery from traumatic rupture of the spleen. One of the most interesting of the older ideas is the "splenoid" theory, largely accredited to Von Stubenrauch. According to this theory, removal of the spleen causes the formation of nodules of tissue resembling spleen by differentiation of previously undifferentiated cells of the peritoneum. Another theory suggested that the nodules represent splenic anlagen which are stimulated to undergo compensatory hyperplasia as a result of splenectomy.

It should be mentioned that one or more accessory spleens are frequent. It has been suggested that they account for the multiple splenic nodules after splenectomy following traumatic rupture. Accessory spleens, however, occur characteristically in the region of the splenic bed and are never widespread as in the case of traumatic implants. Shaw and Shafi, and others, have pointed out that the distribution of splenic implants over the peritoneum, diaphragm, omentum, and in the pelvis is like that observed in implants seen in intra-abdominal malignancy where scattering of viable tumor tissue is presumed. Also, the fact that implants are observed only in cases where the spleen is removed for traumatic rupture, and never after removal of the intact spleen, is good evidence that the nodules observed are autografts of the ruptured spleen.

At this time there can be no doubt that traumatic rupture of the spleen can result in wide distribution of fragments of splenic tissue which may persist as implants over the peritoneum for many years. It would be reasonable to suppose that these autografts, which resemble normal spleen, might be capable of reacting to disease as does intact spleen. It is the purpose of this paper to describe a case which has two features of special interest. First, it is an example of traumatic transplantation of splenic tissue into the soft tissue of the body wall rather than the usual peritoneal location, and, second, the implant after having been present for a considerable length of time, suddenly began to enlarge in what may have been a response to a malarial infection.

CASE REPORT

In August, 1940, J. M. M., colored male, about 52 years of age, was shot through the lower portion of the left chest. The bullet passed through the left lower thoracic wall in an anterior-posterior direction and fractured the ninth and tenth ribs in the posterior axillary line as it passed through the chest wall. There was considerable hemorrhage from the wound of entrance and exit, but never any hemoptysis or positive x-ray evidence of hemothorax, pneumothorax, or pneumoperitoneum. Note is made, however, that breath sounds were not audible over the left lower chest. Study of x-rays made at this time reveal no visible change in the diaphragm which might be construed as evidence of injury. The patient was discharged from the hospital after six days, without operative treatment.

In December, 1941, the patient again appeared at the hospital with a chief complaint of "vomiting blood and pain in stomach." Three weeks before the date of this admission he was kicked by a mule over the left costal margin, anteriorly. He immediately vomited blood-streaked material. He was not incapacitated, however, and worked until one day prior to admission. On this day he lifted a heavy weight and experienced a tearing sensation in his abdomen followed by constant pain and frequent vomiting of blood-streaked vomitus.

Physical examination revealed absence of breath sounds on the left side below the fourth interspace and a tympanitic percussion note over the left lower chest. X-ray examination (after introduction of stomach tube) revealed a large diaphragmatic hernia on the left with herniation of the stomach up to level of the second interspace. Fracture of the tenth rib in the posterior axillary line with considerable displacement of the fragments was also mentioned. The fracture corresponded to that described in August, 1940, as a result of the bullet wound. The ninth rib was well healed, but there had been almost no change in the tenth rib.

The patient was operated upon, and a large rent in the left posterior diaphragm was repaired. The left chest contained transverse colon, two-thirds of the stomach, and the spleen. It is stated in the operative note that the spleen was adherent to the diaphragm and posterior aspect of the parietal pleura near the vertebral column and had to be removed before the hernia could be repaired. Personal communication from the surgeon and a study of the surgeon's notes indicated that the entire spleen was removed and seemed to be intact, although the upper portion was firmly adherent to the diaphragm and posterior portion of the pleura as mentioned above. The surgeon also stated that the spleen was of average size and shape, but that there were many dense, white, somewhat depressed scars across the organ as if lacerations had occurred in the past. After a stormy recovery, he was discharged in good condition on the twenty-third postoperative day. At the time of this admission a note was made that a small, tender swelling was present in the chest wall at the level of the ninth or tenth rib in the posterior axillary line. This nodule appeared to be in the scar of the bullet wound which was received in August, 1940.

The last admission was on Sept. 15, 1942, at which time the patient complained of soreness and gradual increase in size, during the past three or four weeks, of the small mass in the left posterior axillary line which had been noted on the previous admission. He also complained of vague paresthesias along the lower portion of the left chest extending anteriorly to the epigastric region.

Physical examination revealed a firm, ovoid mass about the size of a hen's egg at the level of the tenth rib in the posterior axillary line. X-ray of the area revealed nonunion of the rib at the site of the old injury and little change in the fragments since the time of the original fracture in August, 1940. Clinical opinion was divided as to whether the condition was that of osteomyelitis or malignant or benign neoplasm.

Laboratory findings on this last admission were: hemoglobin, 13.8 Gm.; red blood cells, 4,450,000; white blood cells, 9,800. The differential count was not remarkable.

Upon the day of admission to the hospital, Sept. 15, 1942, the patient had a sudden rise in temperature to 104° F. and a hard chill at 4 P.M. The temperature rapidly fell after the chill and remained normal or slightly subnormal all the following day. On Sept. 17, 1942, at 4 P.M. the temperature again rose above 105° F., and another hard chill was experienced. On this day malarial parasites were demonstrated in the peripheral blood. Tertian ring forms and gametocytes were described. Quinine sulfate was immediately started, and temperature remained normal until the day of operation, Oct. 8, 1942. Questioning of patient elicited the statement that he had an episode thought to be malaria about ten to twelve years ago, but had no chills or fever in recent years and none immediately before admission to hospital.

On Oct. 8, 1942, the tumor mass was excised with a portion of the tenth rib. The rib appeared to be surrounded by the tumor which was described as "soft tumor mass protruding around the rib and in the soft tissues of the chest wall."

During the operation, the pleural cavity was entered, the diaphragm was accidently penetrated, and the peritoneal cavity exposed; but the opening was small and no observations

on the peritoneum were made. The opening in the diaphragm was sutured and the wound was closed.

Postoperative course was not remarkable except for moderate elevation of temperature which began the day of operation and persisted for nine days. A single exception to the above was a chill with temperature of 104° the second postoperative day. The patient was discharged in good condition, Oct. 24, 1942.

Pathologic Examination of the Operative Specimen.—The specimen consisted of a fragment of broken rib with an attached soft tissue mass. The soft tissue measured 3 x 2 cm. in greatest diameters. It did not appear to arise from the rib itself but lay between the ends of the fragments and around the fracture site. The nodule was well encapsulated. Upon gross examination it was described as red, soft, and very friable.

Microscopic examination of the soft mass revealed well-defined splenic tissue. The pulp was not striking in appearance, except for some fibrosis and rather conspicuous sinusoids. Malpighian bodies were numerous and bulky with large germinal centers.

Trabeculae were present in numbers and appearance suggestive of normal spleen. The capsule on the surface was not thickened and resembled a normal splenic capsule.

Beneath the capsule in pulp there were two small areas of hemorrhage showing early organization and containing a few lymphocytes. Scattered throughout the pulp, for the most part in macrophages, were large quantities of brown pigment. All of the pigment was iron-containing, as determined by the ferrocyanide reaction. No noniron-containing pigment was seen. The gross specimen was discarded before photographs or sections of the bone at the fracture site could be made.

Discussion.—The evidence seems to justify the assumption that this is a case of traumatic implantation of splenic tissue in the chest wall at the costophrenic angle.

It seems probable that the bullet in August, 1940, tore through the upper portion of the spleen, lower lateral portion of the left diaphragm at the costophrenic angle, and then passed through the body wall, fracturing the ninth and tenth ribs. In its passage, the bullet may have carried a fragment of splenic tissue through the extreme lower lateral portion of the diaphragm near the attachment to the chest wall and left it lying between the fractured ends of the tenth rib in the body wall where it persisted as an autoplastic transplant, perhaps explaining in part the nonunion of the fracture.

It is possible, of course, that this portion of displaced spleen maintained some attachment to the main mass of splenic tissue for a time, possibly until the rent in the diaphragm healed. It may be, too, that small fragments yet remain trapped in scarred diaphragm and perhaps in the splenic bed. None were described in the course of the operation for repair of the diaphragmatic hernia, however.

While it is not possible to say from either histologic or clinical evidence that the implant increased suddenly in size as result of malaria, it is hard to escape this impression in view of the order of development of events. It is probably safe to assume that this patient's malarial inoculation took place not less than fourteen days prior to his admission to the hospital. Boyd, Stratman-

Thomas, and Muench[1] have pointed out that the gametocytes of *Plasmodium vivax* have not been observed prior to the fourteenth day following inoculation, and may be later in appearance. The rather rapid and sudden increase in size of the splenic nodule may be significant. Coggeshall[4] has shown that the rate of the spleen increase after the first intravenous injection of *Plasmodium knowlesi* in monkeys followed a straight line until twenty-four hours before death. A much greater and more rapidly developing splenomegaly was noted in animals in which a chronic infection existed. Such animals, when injected with large numbers of homologous parasites, showed a rapid increase in size of the spleen as compared with primary infections, followed, however, by a fairly rapid decrease. It is not at all unlikely that the patient in the case described in this paper had chronic malaria.

In this case it should be noted that the splenic pulp, capsule, trabeculae, and Malpighian bodies appear to be like normal splenic architecture in every respect. The various reports in the literature differ somewhat in the description of the autograft nodules as regards the resemblance to normal spleen. One gains the impression, however, that none depart very far from the usual appearance. In one of the cases described in the literature[11] it was noted that, although the splenic pulp, in implants was easily recognized, it was not considered to be identical in detail, and in only two nodules were there structures which might be an attempt at splenic corpuscle formation. Lee[7] indicated that the implants in his case were like normal spleen with pulp and Malpighian bodies. Buchbinder and Lipkoff[2] noted a case in which the transplants resembled normal spleen except for paucity of lymph follicles and trabeculae and an occasional atypical blood vessel relationship. It may be that the degree of similarity of the implants to the normal splenic tissue depends upon the size and components of the original implant. If all components of the spleen are represented in the implant, one might expect it to duplicate the histology of normal spleen. If, on the other hand, only pulp elements in very small fragments are scattered, it would seem possible that other structures might be lacking or scarce in number, although it is believed that reticular cells of splenic pulp are capable of differentiation into Malpighian bodies as well as pulp elements. In most of the recorded cases there has been extensive tearing of the spleen, with hemorrhage, and, presumably, wide distribution of tiny pulp fragments over the peritoneum. In the case described here, however, a large possibly solitary mass is believed to have been displaced. That the presence of all components of adult spleen in implants is not necessary to produce grafts similar to normal spleen is suggested by work of Calder[3] on experimental animals. He observed that in experimental implants all except a narrow rim of tissue at the periphery of the implant becomes necrotic and that the necrotic center is then replaced by proliferation of undifferentiated reticular cells which survive at the edge. He feels that most elements of spleen can be derived from these cells and that the essential factor in growth of splenic grafts is survival and proliferation of reticulum cells.

The possibilities of experimental subcutaneous autografts of spleen have been explored and appear to offer no serious problems. According to Marine and Manley[8] autografts of spleen introduced beneath the subcutaneous fascia

of the anterior abdominal wall in rabbits seldom fail to survive. They state, however, that although an autograft may survive or even grow slightly, it does not show really extensive growth unless the spleen is partially or completely removed. Their transplants which grow or survive are said to have all the general characteristics of normal spleen, both as to number of component structures and their relationship to each other. It appears that no experiments were carried out by them relative to reaction of implants to infection, but they noted that in several cases where animals died of pneumonia, the grafts were congested, and in one case, the transplants were soft, showed increased pulp cells; and they suggest that transplants might react to infections as the intact spleen does. Perla and Gottesman[10] indicate that in many cases, splenic transplants in rats react to protect the animals against *Bartonella muris* anemia. It is interesting that typical tubercles were described in several implants by Shaw and Shafi as an indication that this common finding in intact spleens is also noted in implants. They state, however, that no bilharzial pigment was present in implants, although the patient showed infestation. (It will be recalled that only iron-containing pigment was observed in the case reported in this paper).

It has been suggested by Morrison, Lederer, and Fradkin[9] that accessory spleens remaining after splenectomy may account for some of the failures of operation in certain conditions, such as thrombocytopenic purpura. With this in mind, it would seem probable that implants of spleen might also maintain functional capacity similar to the intact normal spleen, especially when that organ has been removed. It is suggested by the case described here that malaria may be capable of causing enlargement in autografts of the spleen.

Summary and Conclusions.—A case is reported of bullet wound through lower left chest at about the level of the tenth rib in the posterior axillary line. About fifteen months later the patient suffered a rupture of the diaphragm with diaphragmatic hernia; this was repaired about three weeks after the accident. At time of repair the apparently intact spleen was found adherent to the lower surface of the diaphragm and to the posterior chest wall near the spine. It was dissected free and removed to facilitate closure of the rent in the diaphragm. About nine months after operation, a small subcutaneous mass in the left posterior axillary line, which was known to be present in the scar of the bullet wound prior to the operation for repair of the diaphragmatic hernia, increased rather rapidly in size and became painful. Shortly after change in size was noted, the patient developed typical malaria. Operation revealed a mass of characteristic splenic tissue lying in chest wall between the broken ends of the tenth rib. While it is not felt that a positive diagnosis of "malarial" splenomegaly in the autograft of spleen can be made, this possibility is indicated.

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THE BACTERICIDAL ACTIVITY OF SOME DI-(HYDROXYPHENYL) ALKANES*

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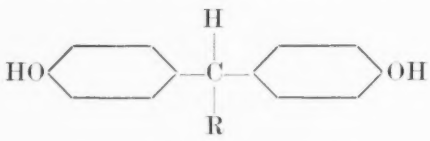
INTRODUCTION

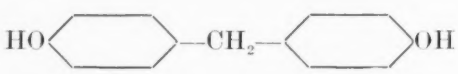
THE bactericidal properties of phenol and phenolic compounds have been the subject of considerable intensive research. On the other hand, bactericidal investigations of series of di-(hydroxyphenyl) alkanes have been somewhat limited.

Harden and Reid[1] studied, among other compounds, a series of the type:



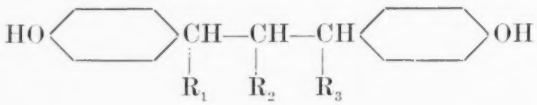
where R may be hydrogen or an alkyl group (from methyl to n-hexyl). Their results showed increasing germicidal activity, against *Staphylococcus aureus*, with increasing length of the aliphatic chain.

Richardson and Reid[2] continued these studies on several α, ω derivatives of the type:



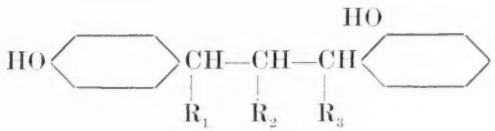
in which the aliphatic chain varied in length from CH_2, as shown, to $C_{10}H_{20}$. These compounds were much less soluble than the corresponding α, α derivatives, and they were able to obtain maximum killing dilutions only for the first four members of the series. Lengthening the aliphatic chain was found to increase bactericidal power against *S. aureus*.

As the result of some organic syntheses, in this laboratory, 23 di-(hydroxyphenyl) alkanes were made available for bactericidal testing. The basic formulas for the compounds discussed in this report, based on the position of the hydroxyl groups, are as follows:



(A)

*From Research Laboratories, Schieffelin and Co.
Received for publication, Aug. 11, 1943.
†Research Bacteriologist.



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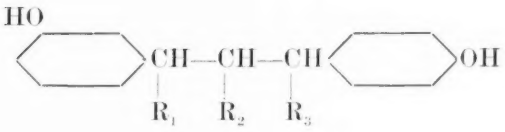
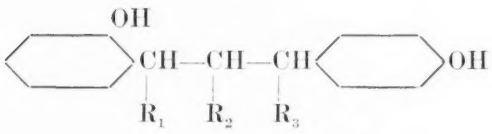


(C)



(D)

R_1, R_2, R_3 may be hydrogen or an organic radical.

For convenience, these compounds are divided into series based on the structure of the aliphatic chain. Series I includes compounds of basic formulas (A), (B), (C), and (D). All the compounds in the other series have the basic formula (A). Chemical investigations of monosubstituted derivatives of 1, 3 di-(p-hydroxyphenyl) propane have been presented by Stuart and Tallman.[3] Chemical studies on the more highly substituted derivatives are to be reported.

METHODS

Stock solutions of the compounds were prepared, in suitable concentrations, in 95 per cent ethyl alcohol, by volume. Dilutions were made from the stock with sterile distilled water. Maximum bactericidal dilutions were obtained in the presence of 20 per cent alcohol, by volume, in all instances, since it is known that the activity of phenolic compounds may be influenced by the concentration of alcohol present.[4] A 20 per cent concentration of ethyl alcohol, by volume, did not kill the test organisms within the time limit used.

The test organisms were *S. aureus 209* and *Eberthella typhosa* grown in F.D.A. nutrient broth. To 5 c.c. of diluted compound, at 20° C. there was added 0.5 c.c. of 18- to 24-hour growth of culture. After standing at this temperature for ten minutes, transfer was made with a 4 mm. loop (No. 23 B. & S. gauge wire) to 10 c.c. of sterile nutrient broth. Subcultures were incubated at 37° C. for forty-eight hours and maximum killing dilutions determined. Tests made for bacteriostatic properties revealed that it was not necessary to further transfer the subculture.

RESULTS

The bactericidal dilutions, against *S. aureus* and *E. typhosa*, for the compounds tested are shown in Table I. It is noted that bactericidal activity against *S. aureus* increases, in the four series of compounds, as the molecular weight of

the alkyl chain increases. With *E. typhosa,* the results are quite different; maximum bactericidal activity in a dilution of 1:8000 is found in Series I with a normal alkyl chain of four carbon atoms in length. Further lengthening of the chain gives compounds, the solubility of which are limiting factors in obtaining bactericidal dilutions for *E. typhosa.*

A given number of carbon atoms have more effect, against *S. aureus,* when substituted as a single chain in the R_2 position (Series I), than when distributed between two chains in the R_1 and R_3 positions (Series II and IV). Substituting the aliphatic chain in the R_2 position (Series III), forming a branched chain arrangement, also did not produce as great an effect as when the chain was in the R_3 position. The addition of carbon atoms in the form of a ring, as in 1,3-di (hydroxyphenyl) 3-phenyl propane, was not as effective as their use in a straight chain.

Branching of the carbon chain, as in 1,3-di (p-hydroxyphenyl) 4-methyl pentane, lowered the bactericidal dilution for *S. aureus* to 1:25000, as compared with 1:40000 for the normal compound, 1,3-di (p-hydroxyphenyl) n-hexane.

In Series I the killing dilutions obtained for *S. aureus* were approximately the same with isomeric ortho, meta, and para derivatives of the same compound.

To show that bactericidal activity is due in large part to the free hydroxyl groups, the di-methoxy derivative of 1,3-di (p-hydroxyphenyl) n-hexane was prepared and tested against *S. aureus.* The ester showed absolutely no killing activity even in saturated solutions; the hydroxyl compound killed the test organism in a dilution of 1:40000.

There seems to be no general relationship between the melting point and bactericidal activity of the compounds tested.

DISCUSSION

It is noted that certain generalizations between bactericidal activity against *S. aureus* and chemical structure, which have been found to hold in the case of other series of phenol derivatives, can also be applied to the series of di-(hydroxyphenyl) alkanes studied. These may be briefly summarized as follows:

1. Increasing the length of the aliphatic chain attached to a hydroxyphenyl ring results in increased bactericidal activity. A given number of carbon atoms has more effect in a single chain than when distributed between two or more chains.

2. Branching of the aliphatic chain lowers the activity below that of the normal straight chain compound.

3. The position of the hydroxyl group on the ring does not materially influence bactericidal activity in unsubstituted isomeric compounds.

The "quasi-specific" character of the compounds tested is evident. The susceptibility of *E. typhosa* reaches a maximum with an alkyl chain containing four carbon atoms. On the other hand, the homologues of higher molecular weight, up to an alkyl chain containing nine carbon atoms, are quite effective against *S. aureus.* This effect has been reported for other phenol derivatives by Klarman and co-workers.[5]

TABLE I

BACTERICIDAL ACTIVITY OF DI-(HYDROXYPHENYL) ALKANES AGAINST *S. aureus* AND *E. typhosa* IN 10 MIN. AT 20° *C.*

BASIC FORMULA	CHEMICAL NAME	SUBSTITUENTS R_1	R_2	R_3	M.P. ° C.	BACTERICIDAL DILUTIONS* S. AUREUS	E. TYPHOSA
	Phenol				43	80	100
	SERIES I						
A	1,3-di (p-hydroxyphenyl) propane	—H	—H	—H	163	3000	4000
A	1,3-di (p-hydroxyphenyl) butane	—H	—H	$-CH_3$	resin	5000	8000
B	1-(p-hydroxyphenyl) 3-(o-hydroxyphenyl) butane	—H	—H	$-CH_3$	resin	5000	7000
A	1,3-di (p-hydroxyphenyl) n-pentane	—H	—H	$-C_2H_5$	99	10000	-†
C	1-(m-hydroxyphenyl) 3-(p-hydroxyphenyl) n-pentane	—H	—H	$-C_2H_5$	resin	12000	-
A	1,3-di (p-hydroxyphenyl) n-hexane	—H	—H	$-C_3H_7$	101	40000	-
B	1-(p-hydroxyphenyl) 3-(o-hydroxyphenyl) n-hexane	—H	—H	$-C_3H_7$	resin	45000	-
C	1-(m-hydroxyphenyl) 3-(p-hydroxyphenyl) n-hexane	—H	—H	$-C_3H_7$	resin	45000	-
D	1-(o-hydroxyphenyl) 3-(p-hydroxyphenyl) n-hexane	—H	—H	$-C_3H_7$	resin	45000	-
A	1,3-di (p-hydroxyphenyl) 4-methyl pentane	—H	—H	$-C_3H_7$	resin	25000	-
A	1,3-di (p-hydroxyphenyl) n-heptane	—H	—H	$-C_7H_9$	resin	70000	-
A	1,3-di (p-hydroxyphenyl) n-octane	—H	—H	$-C_8H_{11}$	resin	150000	-
A	1,3-di (p-hydroxyphenyl) 3-phenyl propane	—H	—H	$-C_6H_5$	105	15000	-
	SERIES II						
A	2,4-di (p-hydroxyphenyl) pentane	$-CH_3$	—H	$-CH_3$	143	<5000	-
A	3,5-di (p-hydroxyphenyl) n-heptane	$-C_2H_5$	—H	$-C_2H_5$	resin	45000	-
A	4,6-di (p-hydroxyphenyl) n-nonane	$-C_3H_7$	—H	$-C_3H_7$	resin	90000	-
	SERIES III						
A	1,3-di (p-hydroxyphenyl) 2-methyl propane	—H	$-CH_3$	—H	130	<5000	-
A	1,3-di (p-hydroxyphenyl) 2-ethyl propane	—H	$-C_2H_5$	—H	102	10000	-
A	1,3-di (p-hydroxyphenyl) 2-propyl propane	—H	$-C_3H_7$	—H	118	18000	-
A	1,3-di (p-hydroxyphenyl) 2-ethyl n-hexane	$-C_3H_7$	$-C_2H_5$	—H	107	80000	-
	SERIES IV						
A	2,4-di (p-hydroxyphenyl) n-hexane	$-CH_3$	—H	$-C_2H_5$	resin	15000	-
A	2,4-di (p-hydroxyphenyl) n-heptane	$-CH_3$	—H	$-C_3H_7$	resin	35000	-
A	3,5-di (p-hydroxyphenyl) n-octane	$-C_2H_5$	—H	$-C_3H_7$	resin	70000	-

*The figures indicate the volume to which one gram of compound can be diluted with 20 per cent ethyl alcohol by volume and still retain bactericidal properties.

† – indicates that the compound is not bactericidal in a concentration totally soluble in 20 per cent ethyl alcohol, by volume.

It is interesting to note that the series of compounds tested have other physiologic properties in so far as they are able to bring about estrogenic responses.[6]

SUMMARY

Twenty-three di-(hydroxyphenyl) alkanes have been tested for bactericidal activity. In general, they exhibit increased killing action, in vitro against *S. aureus*, with increasing length of the alkyl chain. The susceptibility of *E. typhosa* is at a maximum with the n-butyl derivative. Generalizations which have been applied to other series of phenols, regarding the relation of bactericidal activity to chemical structure, are found to be valid for the series of compounds tested.

Grateful acknowledgement is made of the advice and encouragement given by Dr. R. C. Tallman, Director of Research, in this problem and also for supplying the compounds tested.

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A STUDY OF OILS USED FOR INTRAMUSCULAR INJECTIONS*

A Study of the Physical, Chemical, and Biologic Factors

Willis E. Brown, M.D., Violet M. Wilder, Ph.D., and
Pauline Schwartz, A.B., Omaha, Neb.

ALL physicians active in the clinical practice of medicine find it convenient to administer drugs in oil by intramuscular injection. With the increasing use of water-insoluble drugs, this route of administration is becoming more frequent. While it is true that most of the oils placed on the market at the present time are fairly satisfactory, all of us sooner or later find patients who react unhappily to some of them. Because of such an experience in our own clinic, we undertook to investigate the factors which should guide a physician in his selection of the oil vehicle for such medication.

The ideal oil should meet the following requirements:

Chemically it should be a stable oil, neutral in reaction, i.e., containing no excess acid. It should not react with the medication to form toxic degradation products.

Biologically it should be inert and nonirritating. This oil should be essentially free of antigenic properties and be rapidly absorbed from living tissue leaving no residue.

Physically it should be a good solvent or dispersing medium. It should not be too viscid to pass readily through the needle. The extremes of temperature encountered in sterilization (200° F.) and freezing (–10° to –20° F. of doctor's car in winter) should not materially alter any of the above features.

In searching through the literature, very little information is available regarding these data on the oils in common clinical use. Most of the articles concern themselves with specific examples of allergic reaction or hypersensitivity to some particular oil. While these cases are not too uncommon, they are a specialized group of reactions and were not considered in this study.

Emery et al.[1] working with rats studied the absorption of stilbestrol and theelin from intramuscularly injected sesame, peanut, castor, and olive oils. They found oil retention cysts in these rats ranging in size from microscopic to 1 or 2 mm. in size. Emery et al. found that oils differed somewhat in the cyst formation which they incited, and these authors recorded the following order of increasing tissue reaction: peanut, olive, sesame, and castor.

Deanesly and Parkes[2] studied the absorption of oil following subcutaneous injection. They report that in rats and mice, olive oil is absorbed most rapidly, that sesame and castor oil are absorbed with increasing difficulty, and that castor

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This study was carried on by a grant from Smith Dorsey Company, Lincoln, Nebr., who prepared and made available the oils used in this study.

oil remains in tissue almost indefinitely. Chart 1, modified from these authors, graphically presents their data.

These two reports suggest that castor oil produces the most marked reaction and persists longer than other oils and that olive and sesame oils are the better oils for clinical use.

Dr. E. A. Sharp[3] has made available to us, through personal communication, the results of observations on the inflammatory reaction produced by the intramuscular injection of olive, sesame, and peanut oils. Chart 2 is prepared from such data and shows that olive and sesame oils produced the least inflammatory changes in muscle tissue and were the most readily absorbed. Peanut oil produced the most marked inflammatory change.

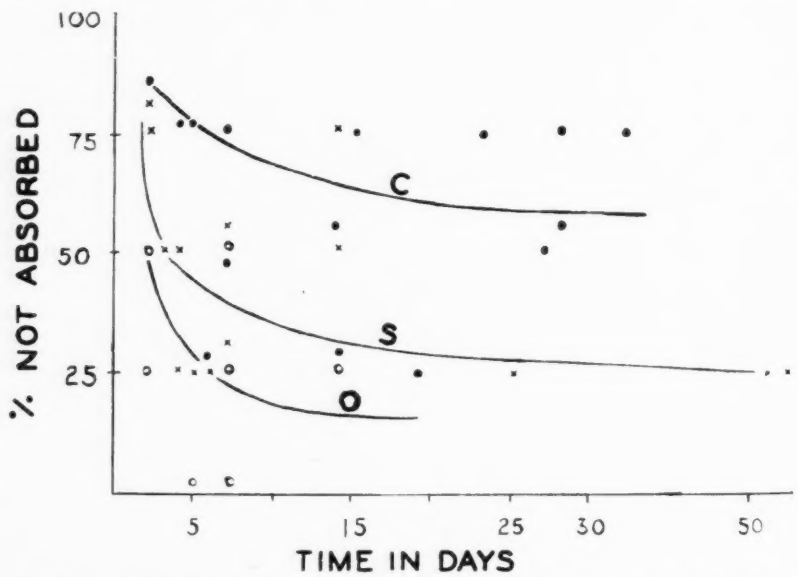


Chart 1.—The absorption of oil from subcutaneous injection. Arranged from data published by Deanesly and Parkes.[2]
Olive oil o Sesame oil X Castor oil •

METHOD

Because of the scarcity of material in the medical literature on this subject, we set up a project designed to assist us in the selection of the best oil for intramuscular injection. Four commonly used oils (corn, cotton seed, sesame seed, and peanut) were selected for study. Samples of these oils and their chemical and physical properties were made available to us through the courtesy of Dr. F. S. Bukey.

The antigenic properties were investigated in the following manner. Twenty patients received at weekly intervals two injections of each of the four oils to be considered. Approximately two months later these patients were tested by the patch and intracutaneous test techniques according to standard specifications.[4]

The biologic reaction was investigated by injecting oils intramuscularly into the large extremity muscle of experimental animals (rats and rabbits). Every

possible care was taken during the injection of the oil to obtain satisfactory antisepsis and to deliver as nearly as possible pure sterile oil. Injections were made at right angles to the surface, as near the center of the muscle as possible, and the area was gently massaged. These animals were sacrificed at varying time intervals of from one to nineteen days. The muscle was dissected free and fixed in 10 per cent formalin. The tissue was then cut, and frozen sections were prepared for fat stain with scharlach R. The balance of the muscle was then run through dehydrating alcohols, xylol, into paraffin sections and stained with hematoxylin and eosin.

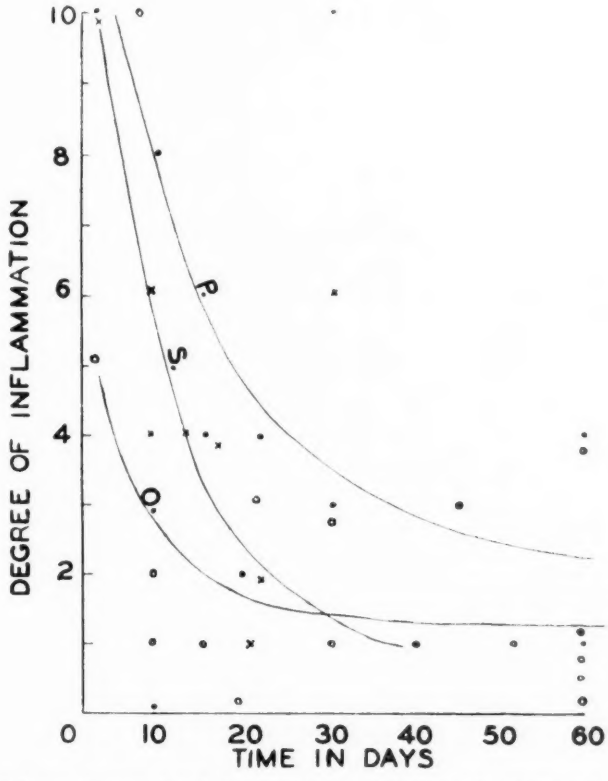


Chart 2.—Inflammation induced in muscle by injected oil. Arranged from data furnished by Dr. Sharp.[3]

Olive oil o Sesame oil X Peanut oil •

RESULTS

In Tables I, II, and III will be found a summary of the data observed. The chemical and physical properties of these oils are shown in Table I. Corn oil showed slightly less viscosity than the other oils at room temperature but was definitely less viscid at 3° C. Corn oil remains fluid down to temperatures of −10° to −15° C. (solidification point). This becomes significant for physicians who carry their bags in their cars in the winter time. All of the oils tolerated sterilization temperatures satisfactorily, smoking temperatures being above sterilization temperatures in all cases. The acids found in these oils are the result of hydrolysis. There is considerable variation of the acid value in different

samples of each oil. Apparently the source and handling of the material are more important than the type of oil when this factor is considered. Several pharmaceutical laboratories have added chemical agents as preservatives, either as bacteriostatics or antioxidants. Some of these agents have proved quite irritating clinically. Hydroquinone was found as an antioxidant in one manufacturer's product. This was effective in preventing the deterioration of the active ingredient, but proved very irritating clinically in some cases, especially if a small amount of oil was deposited subcutaneously.

TABLE I

THE PHYSICAL AND CHEMICAL PROPERTIES OF THE OILS TESTED

		COTTON SEED	SESAME SEED	CORN	PEANUT
"SMOKING TEMPERATURE" (C°)		235°	212°	243°	220°
Solidification Point (C.°)		−12° to −13°	−4° to −16°	−10° to −20°	−3°
Specific Viscosity (Oil/Water)	23° C.	33.9	31.8	29.7	35.5
	3° C.	82.7	71.6	63.9	Semisolid
Acid Value		2.5 to 7.3*	3.0 to 1.4	2.5	0.12 to 0.3

*One sample had an acid value of 21.6.

TABLE II

PATIENTS GIVEN TWO SENSITIZING INJECTIONS OF A POOLED SAMPLE OF ALL FOUR OILS AT ONE WEEK INTERVAL. SIX TO EIGHT WEEKS LATER THEY WERE TESTED FOR SENSITIVITY BY PATCH AND INTRACUTANEOUS INJECTION OF EACH OIL, AND THE REACTION READ AT 24°, 48°, AND 72°.

PATIENT	COTTON SEED			CORN			SESAME SEED			PEANUT		
	24°	48°	72°	24°	48°	72°	24°	48°	72°	24°	48°	72°
L	−	+	+	−	−	−	−	−	−	+	+	+
C	−	−	−	−	−	−	−	−	−	−	−	−
U	−	−	−	−	−	−	−	−	−	−	−	−
S	−	−	−	−	−	−	−	−	−	−	−	−
G	−	−	−	−	−	−	−	−	−	−	−	−
K*	−	+	+	−	−	−	−	−	−	−	+	+
T	−	−	−	−	−	−	−	−	−	−	−	−
D	−	−	−	−	−	−	−	−	−	−	−	−
H	−	−	−	−	−	−	−	−	−	−	−	−
C	−	−	−	−	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−	−	−	−	−

*On fifth day (120 hrs.) there was erythema about all sites of injection.

Of the twenty patients studied for the antigenic effect of these oils, three complained of pain on the second sensitizing injection; the others offered no complaint. Patch and intracutaneous tests were completed on eleven patients, and the results are found in Table II. Reactions in general were uncommon. Definite conclusions cannot be drawn from this small series, but it would appear that sesame and corn oil are less antigenic than the cotton seed and peanut oils.*

On studying the tissues microscopically, we observed a typical trend of reactions. Within twenty-four hours there was a moderate increase in round cells and the oil seemed to be diffusely scattered through the muscles. It had

*No attempt is made to study the mechanism of this sensitization. It has been suggested that it results from small amounts of protein in the oil.

Fig. 1.

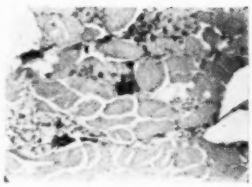


Fig. 2.

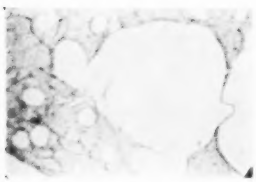
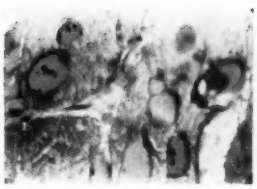
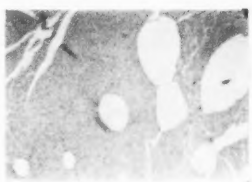
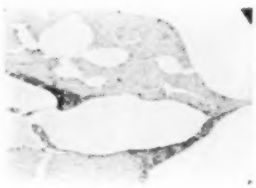


Fig. 3.







A. *B.* *C.*

Fig. 4.

Fig. 1.—Peanut oil reaction seen at 24°—low power.
Fig. 2.—Corn oil reaction seen at five days—low power.
Fig. 3.—Fat stain—peanut oil 4 days—low power.
Fig. 4.*A*, Sesame oil at ten days, low power.
B, Peanut oil at ten days, low power.
C, ibid., high power.

TABLE III

THE MICROSCOPIC STUDY OF TISSUE REACTION TO INTRAMUSCULARLY INJECTED OIL

DAY	COTTON SEED	SESAME SEED	CORN	PEANUT
		RABBIT		
1	Many leucocytes. Oil along fascial planes. (No cysts in spec.)	Oil in cysts and along fascial planes. Very few leucocytes.	Oil in cysts. Very few leucocytes about cysts.	Oil in cysts. Many leucocytes.
4	Oil along fascial plane and in cysts. Leucocytes about some of the cysts.	--	Oil in cysts. No leucocytes.	Some of cyst walls very thick. Mod. leucocytes.
11	Oil cysts. Moderately thick walls. No leucocytes.	Thin-walled cysts.	Very thin-walled oil cysts. No leucocytes.	Some of cyst walls very thick. Many leucocytes.
14	Thick-walled cysts. No leucocytes.	--	Very thin-walled oil cysts. No leucocytes.	Some of cyst walls very thick. No leucocytes.
		RAT		
1	Oil beginning to be encysted. Few leucocytes.	Very few leucocytes. Oil dispersed.	Very few leucocytes. Oil dispersed.	Many leucocytes, edema. Oil dispersed.
4	Mod. encystment. Wall mod. thick. Some leucocytes.	Oil encysted. Thin walls. Few leucocytes. No edema.	Clear cysts with very thin walls. Few leucocytes.	Part of oil encysted. Heavy leucocytes and edema. Some muscle necrosis. Wall surrounded by leucocytes.
10	Encysted oil. Mod. thick wall. Few leucocytes.	Cyst wall mod. thick. Mod. leucocytic reaction.	Thin-walled cysts. Leucocytes nearly gone. Slight reaction.	Oil cysts persist with heavy leucocytes and edema.
14	Oil cysts persist and leucocytes mod.	Small thin-walled oil cysts. Very few leucocytes.	Cysts smaller. Leucocytes gone. Cyst walls very thin.	Similar to above. Sl. less leucocytic reaction.
19	Oil cysts persist with mod. thick wall. Few leucocytes.	Thin-walled cysts. Mild leucocytes.	No leucocytes. Few fibroblasts in cyst wall.	--
Severity of reaction	3	2	1	4

a tendency to accumulate along fascial sheaths, but was also distributed between muscle bundles. (Fig. 1). In two or three days the oil tended to accumulate into small droplets, and around these droplets appeared a layer of fibrin with a large epithelioid type of cell. The accumulation of leucocytes and wandering cells was peripheral to this oil cyst (Fig. 2). Fig. 3 is of tissue prepared for the demonstration of fat and is stained with scharlach R; this demonstrates that these spaces are actually oil cysts. The leucocytic reaction reached a peak between days 3 and 6 and then tended to subside slowly. The rate and intensity of these changes varied in the different oils. In Table III are recorded our observations. Based on these factors we have attempted to grade the tissue reaction to these oils. In general, all of the oils formed many microscopic cysts throughout the involved muscle. As noted in this chart, there is a qualitative and quantitative difference in response. Fig. 4 compares the microscopic picture of corn oil and peanut oil as seen at ten days.

In general, corn and sesame oil produced the least amount of reaction, as judged by the amount of fibrin deposited, and leucocytic infiltration for the

shortest period of time. The persistence of oil cysts could not be determined from this short period of observation; they did, however, seem to be smaller in corn and sesame oil animals. Peanut oil produced the most marked reaction; and cotton seed oil was in between these, showing somewhat greater variation from animal to animal.

CONCLUSION

1. The criteria for selecting an ideal oil for intramuscular injection have been suggested. These include physical, chemical, and biologic specifications. Using these criteria, corn, peanut, sesame seed, and cotton seed oils were studied.

2. The physical and chemical properties of these oils as they apply to their use in medicine have been reviewed and evaluated.

3. The antigenic properties of these oils were tested; sesame seed and corn oil did not produce sensitivity by this technique, while cotton seed and peanut oil produced reactions.

4. The reaction of muscle to these oils was studied, and the following factors have been used in evaluating the intensity of this reaction: (a) accumulation of leucocytes, (b) formation of oil cysts, and (c) disposition of fibrin.

5. By these criteria *sesame* and *corn* oil are superior to peanut and cotton seed oil for intramuscular injection, for they are: (a) more suitable physically and chemically for this purpose, (b) more quickly absorbed from tissue, (c) less antigenic, and (d) less irritating to tissue.

We wish to express our appreciation to Dr. J. P. Tollman, Professor of Clinical Pathology, who assisted in evaluating the tissue reaction.

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BARBITURATE INTOXICATION AND PICROTOXIN TREATMENT

Presentation of a Case

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DESPITE lack of complete information, reports on barbiturate poisoning indicate that it is a problem of important magnitude. Hambourger,[1] at the request of the Council on Pharmacy and Chemistry of the American Medical Association, has made a study of the promiscuous use of the barbiturates. He concluded:

1. More than 1,200,000,000 grains of barbituric acid derivatives were sold in the U.S.A. in 1936.

2. The number of suicidal deaths by the barbiturates in the U.S.A. in 1936 was 300.

3. The number of suicides by barbiturates has shown a definite upward trend during the past decade.

Statistical reports of the Medical Examiner's Office of the City of New York revealed the number of suicides by barbiturates, accidental and undetermined, in the City of New York alone, to be 32 cases for the year 1937; 42 cases for the year 1939; 48 cases for the year 1940; and 60 cases for the year 1941. Accurate statistics as to the number of persons poisoned who did not die are not available, as physicians do not report those cases.

Perusal of foreign literature reveals that the use of barbiturates is as common abroad as in the U.S.A.[2] Thus the widespread use of barbiturates by the layman has inevitably led to many instances of accidental overdosage or suicidal poisoning. It is therefore imperative that the general practitioner, as he is the one who is first called, be familiar with the symptoms of acute intoxications, as well as cognizant of a suitable and energetic antidote.

The symptoms of barbiturate poisoning vary with the amount of drug taken. When absorption is gradual, confusion, ataxia, difficulty in swallowing, vomiting, and motor excitement occur. Diagnosis is apt to be difficult. Any patient found in deep coma, with shallow rapid respiration, fixed miotic pupils, corneal reflexes abolished, absent reflexes, thready pulse, and low blood pressure, should suggest the possibility of barbiturate poisoning. Energetic therapy is essential; the aim of the treatment is to combat the action of the drug by antagonistic drugs and supportive therapy. Table I contains approximate fatal doses for the commoner barbiturates.[3]

Picrotoxin is the active principle of several plants, notably *Anamirta paniculata* or "fish berries."[3] Its use as an antidote in barbiturate intoxications was first suggested by Maloney, Fitch, and Tatum[4] on the basis of animal experiments. Picrotoxin gained the attention of the clinicians as a result

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of the pioneer work and studies of Maloney and his associates,[5] and Linegar and his co-workers.[6] Murphy et al.[7] reported the first clinical attempt to administer picrotoxin in adequate doses in acute barbiturate poisoning. A number of clinical cases[8-12] have been reported which seem to substantiate the animal evidence that picrotoxin is an effective physiologic antidote for barbituric acid intoxications in human beings.

Picrotoxin stimulates the medullary centers and cortical centers. The respiratory and vasomotor centers and the autonomic centers are also stimulated. There is a hyperactivity of the skeletal muscles and some myoclonic movements. In toxic doses, it causes nausea, vomiting, cold sweat, diarrhea, slow pulse, clonic convulsions, and paralysis. Picrotoxin, injected intravenously, disappears rapidly from the blood and could not be demonstrated there or in the liver or muscles after two hours.[13]

It has a narrow margin of safety. However, clinical evidence indicates that in barbiturate poisoning much higher doses may be used. It should be administered by divided doses. The amount and interval should depend upon the response of the patient. The immediate object is to maintain adequate respiratory and circulatory function by the stimulant. Bleckwenn et al.[14] in a series of clinical observations found approximately one mg. of picrotoxin was an antidote for approximately 30 to 40 mg. of pentobarbital sodium.

TABLE I

COMPOUND	SEVERE INTOXICATION, BUT GENERALLY WITH RECOVERY	GENERALLY FATAL
Allonal	10 Gm.	More than 15 Gm.
Amytal	1.5 to 2 Gm.	2 to 3 Gm.
Barbital	3 to 10 Gm.	5 to more than 20 Gm.
Dial	2 to 2.5 Gm.	More than 2.5 Gm.
Nembutal (Pentobarbital Sodium)	More than 1 Gm.	More than 2 Gm.
Pernocton	0.5 to 1 Gm.	More than 1 Gm.
Phanodorm	1.2 Gm.	More than 10 Gm.
Phenobarbital	4 to 7 Gm.	6 to 9 Gm.

It is the purpose of this communication to report a case of pentobarbital sodium (nembutal) intoxication treated by picrotoxin with recovery. The patient swallowed 3 grams of the drug which is considered a lethal dose.

CASE REPORT

The patient, a 24-year-old nurse, was first seen in deep coma at 8 P.M. on May 13, 1943. Her color was dusky, pupils miotic and nonreacting. All her reflexes, including the corneal, were absent. Her heart rate was rapid and sounds were of feeble quality; the pulse small, rapid, and thready, rate 124 per minute; the blood pressure 90/50. The respiratory rate was 30 per minute, labored but not stertorous; temperature 96.4° F.

The family revealed she had threatened suicide. Later, on recovery, patient verified that she had swallowed three grams of nembutal at about 4 or 5 P.M. that afternoon.

May 13, 1943, *Treatment Administered:*

(a) *The immediate treatment:*

1. Intravenous injection of 1.5 c.c. size coramine ampules every fifteen minutes for six doses, followed by
2. Six intramuscular injections of metrazol ampules (1 c.c.) at half-hour intervals.
3. Gastric lavage

4. Body heat applied

5. Change of patient's position with removal of tracheal secretion.

The results with the therapy were negative, and the patient's condition remained critical. It was felt that we were dealing with a severe case of deep barbiturate poisoning, and therefore treatment with picrotoxin was instituted at 1 A.M., i.e., about five hours after first seeing the patient.

(b) *Picrotoxin Therapy:*

May 14, 1943, 1 A.M.: 2 c.c. of a 0.3 per cent (6 mg.) solution of picrotoxin was given intravenously.

1:15 A.M.: 2 c.c. of 0.3 per cent (6 mg.) solution of picrotoxin was given intravenously.

1:30 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

1:45 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

2 A.M.: 1 c.c. of 0.3 per cent (3 mg.) solution of picrotoxin was given intravenously.

The patient at this time began to manifest restlessness, winced when her skin was pinched hard, and dribbled her urine. Temperature rose to 98.2; pulse rate diminished to 110; blood pressure 100/60; respiratory rate was 24.

Picrotoxin was continued at half-hour intervals. During this time other adjuvant measures, such as (1) continued body heat, (2) maintenance of open airway; and (3) changing of patient's position, were constantly carried out.

May 14, 1943, 6 A.M.: Corneal reflexes returned; there were twitchings of the tongue and lips. Heart sounds improved, the pulse rate was 100 and of good quality. The lungs were clear; lips were dry but less cyanotic. Injections with picrotoxin were continued at 1 c.c. doses intramuscularly at 45-minute intervals.

7 A.M.: Temperature began to rise and reached 100.6; pulse rate increased to 120, but was of good quality; and the respiration was 29. Lungs were clear. It was felt that the patient's temperature was due to dehydration.

8 A.M.: Temperature was 101.2; pulse rate, 138; and the respiratory rate, 28.

Patient showed increasing restlessness; blood pressure rose to 120/70; heart sounds were of good quality; and picrotoxin was continued intramuscularly at 45-minute intervals in 1 c.c. doses. Pupils reacted to light and were of normal shape.

9 A.M.: 2000 c.c. of 5 per cent glucose in saline intravenous clysis, high colonic irrigation, followed by retention enema of warm coffee.

11 A.M. to 2 P.M.: Reflexes returned and coma seemed less severe.

1. Continued 1 c.c. of picrotoxin intravenously at 45-minute intervals.

2. Continued intravenous drip at the rate of 30 drops per minute.

2 P.M.: Patient vomited green mucus, followed by loose bowel movement; temperature fell to 100.6, pulse rate was 100, and the respiration rate was 20. Patient grunted and moved when pricked.

At this time it was decided to stop picrotoxin injections with the view of ascertaining whether recovery would continue without analeptic medication. Caffeine-sodio-benzoate ampules, 0.5 grams intramuscularly, was substituted at half-hour intervals.

4:30 P.M.: A definite depression was noted, patient became quite immobile, respiration labored, and the pulse rate rose to 119; the temperature became normal, 98.6.

One c.c. intravenous injection of picrotoxin was resumed at 15-minute intervals for two doses. Remission was immediate, twitchings were noted; there was spontaneous bowel evacuation with vomiting of bile.

5 P.M., May 14, 1943, to 4 A.M., May 15, 1943: Picrotoxin continued intramuscularly at 45-minute intervals.

May 15, 1943, 4 A.M.: Patient showed convulsive movement of head, and legs began rolling from side to side. Picrotoxin was stopped.

4 A.M. to 5:45 A.M.: No medication. Patient quiet, physical condition good.

5:45 A.M.: Picrotoxin given intramuscularly at 1 hour intervals for two doses.

Patient became active, kicking covers off body, lifting head, waving arms and grunting.

8 A.M.: Patient began to cry, continued to kick and roll in bed. Stopped picrotoxin therapy. Color good, pulse and heart rate of good quality, rate 90 per minute.

9 A.M.: Patient uttered indistinct sounds; enunciation slow; speech thickened, unintelligible; crying; still unconscious. No medication.

12 M.: Intervals of lucidity and crying spells; quiet sleep; restless muttering; sounds more distinct; speech less slurred.

4 P.M.: Patient perfectly lucid, fully recovered except for weakness.

COMMENT

In the case presented certain facts emerge and are worthy of comment. The physical signs indicated severe poisoning from barbiturates. The amount of drug taken was within the range considered fatal. Although high doses of metrazol and coramine were employed in the beginning of therapy, the results were negative. The intravenous use of picrotoxin (21 mg.) in one hour elicited a marked awakening phenomenon. After thirteen hours of continuous picrotoxin therapy, with definite improvement in the patient's condition, it was decided to stop analeptic therapy. The patient was examined two and a half hours later, and definite depression was noted, the pulse rate increased, respiration became labored, etc. Renewal of intravenous injections of picrotoxin brought about immediate remission.

The Council on Pharmacy and Chemistry[15] stated the following:

"The cautious use of picrotoxin in barbiturate poisoning would therefore seem justifiable in cases which can be carefully studied with the view that they may supply sufficient accurate data for which the proper place of picrotoxin as an antidote may be established."

No pretense is made to recommend picrotoxin as the sole antidote in barbiturate poisonings. Mild cases of barbiturate poisoning need not be treated with picrotoxin. There are accumulated sufficient experimental and pharmocologic evidences to establish picrotoxin as an active antidote for patients profoundly depressed by barbiturate poisoning. The present case and a number of clinical cases which have been reported seem to substantiate the experimental animal evidence.

SUMMARY AND CONCLUSIONS

1. Statistics reveal a widespread increase in the use of barbituric acid derivatives and a resultant rise in the number of poisonings, intentional or accidental.

2. The diagnosis of barbiturate poisoning is difficult; the symptoms are manifold. The presence of coma with shallow rapid respiration, contracted pupils, absent or diminished reflexes, and feeble and rapid pulse should raise the possibility of barbiturate poisoning.

3. A case of pentobarbital sodium poisoning is reported. The patient was treated by injections of picrotoxin. She received 135 mg. of the drug. The patient recovered after swallowing 3 grams of the drug, considered within the range of a lethal dose.

4. Metrazol and coramine were employed in the beginning of therapy, but there were no clinical signs of improvement until picrotoxin was administered.

5. It may not be amiss to mention that in this instance picrotoxin could not be purchased, although practically every pharmacy in the neighborhood was

called. The drug had to be borrowed from a hospital. It is therefore suggested, in view of increasing barbiturate use in our country, that picrotoxin be stored by druggists.

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COLD HEMAGGLUTINATION REACTIONS IN TUBERCULOSIS*

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THE diagnosis of primary atypical pneumonia of unknown etiology is frequently difficult and uncertain. The signs, symptoms, clinical course, and x-rays may vary from case to case, and from season to season. Recently Peterson, Ham, and Finland[1] suggested the possibility that the presence of cold autohemagglutinins might serve as a diagnostic aid in some forms of the disease. However, more extensive research is indicated to establish whether a titer exists above which the sera of patients with atypical virus pneumonia would invariably agglutinate their own cells, and below which no other serum would demonstrate the same phenomenon.

Horstmann and Tatlock[2] investigated the problem still further and employed isohemagglutination methods, using washed type "O" cells. They reported a high incidence of cold isoagglutinins of significant titer in the serum of patients with atypical pneumonia. Their control patients were invariably negative for cold agglutinins.

The present investigation is an attempt to discover the degree of specificity of cold autoagglutinins and isoagglutinins in random control hospital and clinical patients, as well as to study these reactions in patients with early and late tuberculosis. The latter group was chosen because it not only represented another form of pneumonitis, but a form which, in its early stages, conceivably might be confused with atypical pneumonia.

Eighty-two proved cases of tuberculosis were studied. Thirty-three were at the time untreated patients admitted to the tuberculosis wards. Forty-nine were ambulatory, receiving pneumothorax treatment in the outpatient clinic. The ward cases consisted of fourteen with symptoms of six months' duration or less, considered early cases, and nineteen with symptoms of more than six months' duration, considered chronic cases. Six of the former group had symptoms of less than one month duration or no symptoms at all. A control group of eighteen patients from an acute medical ward and twenty-four patients chosen at random from the outpatient clinic were included.

METHOD FOR COLD ISOAGGLUTINATION TEST

1. *Preparation of Washed Type "O" Cells.*—A sample of six c.c. of fresh cells from a Blood Bank Flask from which the plasma had been siphoned or ten c.c. of blood from a type "O" donor mixed with 1 c.c. of 2.5 per cent sodium citrate was used. It was centrifuged at 1,500 revolutions per minute for five minutes. The supernatant fluid and layer of white cells were removed by siphon. Twenty-five c.c. of sterile normal saline (0.85 per cent) were added and the mixture was centrifuged again for five minutes. This was repeated three times. If hemolysis occurred, the sample was discarded. The cells were

*From the Biochemical Laboratories, Kings County Hospital, W. W. Hala, Director of Laboratories.

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packed down well, and a 2 per cent suspension was made by diluting 2 c.c. of the washed cells to 100 c.c. of sterile normal saline solution. The unwashed cells keep for about three days in the refrigerator. The cell suspension must be kept in a refrigerator and may be used until hemolysis occurs.

2. *Procedure for Cold Isoagglutination Test.*—One c.c. of the patient's clear unhemolyzed serum was placed in a test tube containing 3 c.c. of normal saline and mixed. One c.c. of the diluted serum (1:4) was transferred to a small test tube, and 0.1 c.c. of the freshly prepared 2 per cent suspension of human cells was added. The tube was then placed in an ice bath kept in a refrigerator at 1 to 4° C. Readings were taken in six to twelve hours, and the tubes were kept in the ice bath until ready to read. The above procedure was repeated with 1 c.c. of 1:160 and with higher dilutions of the serum. The results were reported as follows:

A. Negative: If on inverting the tube three times, a homogeneous suspension of cells is present.

B. Doubtful: If on inverting the tube three times, very fine particles are present, but disappear within thirty seconds to give a suspension similar to A.

C. Positive: If on inverting as above, there are present many fine persistent particles (1+) to a blood disc which is loosened from the bottom of the tube and which breaks into large coarse particles (4+).

In order to confirm that the positive reactions were typical cold agglutinins, the tubes were allowed to remain at room temperature for an hour or until the agglutinations disappeared. The tubes were replaced in an ice bath in the refrigerator for from two to six hours. The agglutinations always reappeared.

PROCEDURE FOR COLD AUTOAGGLUTINATION TEST

One c.c. of clear unhemolyzed serum to be tested was placed in a small tube. The tube containing the clot was inverted several times and 0.05 c.c. of the suspension of the patient's cells was added to the serum tube. The tubes were placed in an ice bath as described under isoagglutination, and the results were read in the same way.

COMMENT

In no instance was a positive isoagglutination obtained in a titer of 1:160 or over. One patient in the early tuberculosis group showed a positive test, but no patients with symptoms of one month or less demonstrated a positive reaction. In the control group of ward patients the diagnoses of those reacting positively were hypertensive cardiovascular disease, undiagnosed gastrointestinal disease, and nonspecific colitis. No history of virus pneumonia or tuberculosis was obtained from any of these patients. No positive tests were found in the outpatient clinic control group.

RESULTS

The results are reported in Table I.

The present series is not large enough to draw final conclusions concerning the relative correlation of cold autoagglutinins and isoagglutinins with early

and late cases of tuberculosis. It is interesting that no high titers were found in this series, but it is impossible to state with certainty as yet that high titers do not exist in some stage of the tuberculosis disease process. If further investigation of large numbers of patients reveals that those with pneumonitis other than atypical pneumonia invariably fail to develop cold agglutinins of high titer, the test will prove to be of great value in the differential diagnosis of virus pneumonia.

TABLE I*

PATIENTS	SEX	NUMBER	POSITIVE ISOAGGLUTININS (1:4 DILUTION)		POSITIVE AUTOAGGLUTININS	
			NUMBER	PER CENT	NUMBER	PER CENT
T.B. Wards	M	23	6	26	12	52
	F	10	3	30	6	60
	M+F	33	9	27	18	55
T.B. Clinic	M	26	3	12	9	38
	F	23	5	22	14	61
	M+F	49	8	16	23	47
Control						
Clinic	M+F	24	0	0	2	8
Ward	F	18	3	17	5	28
Total	M+F	42	3	7	7	17

*The tuberculosis cases were studied through the courtesy of Dr. C. E. Hamilton, Director of the Tuberculosis Services at Kings County Hospital.

It was also noted that not only did autoagglutinins occur more frequently in all groups than did isoagglutinins, but that there was a definitely larger number of positive autoagglutinin tests obtained in the tuberculosis groups than in the control group. More extensive work is indicated to discover whether there is any clinical significance to this observation, or whether this is merely an example of the rather nonspecific nature of cold autohemagglutinins.

CONCLUSIONS

Cold isohemagglutination and autohemagglutination tests were performed on a series of tuberculosis patients. In none of those showing a positive cold isoagglutination reaction was a high titer noted. This suggests that the cold isoagglutination test previously reported[2] may be of value in the differential diagnosis of some forms of early tuberculosis from atypical pneumonia. Since positive reactions were found in low titer in tuberculosis patients as well as in three control patients it should be emphasized that in order that a test be considered positive for atypical pneumonia, the cold agglutinins must be present in high titer.

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PURPURIC LESIONS IN MENINGOCOCCIC INFECTIONS*

Diagnosis From Smears and Cultures of the Purpuric Lesions

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THE laboratory diagnosis of meningococcus infections has assumed added importance due to the congestion of populations in industrial and camp areas during the war emergency. We have had the opportunity to study a series of 182 cases at the Station Hospital, and the purpose of this report is to record our experiences in culturing the spinal fluid and blood of these patients and to show the value of smears and cultures from the purpuric lesions as an aid in early diagnosis of meningococcus bacteremia.

After considerable experimentation, we developed an efficient culture medium which has enabled us to obtain consistently profuse cultures of the meningococcus after an average incubation period of eighteen hours. In many cases colonies have been observed after only ten hours' incubation. We have also used this medium with remarkable success in the culture of the gonococcus.

DEXTROSE-STARCH-CHOCOLATE-AGAR FOR CULTURE OF MENINGOCOCCI

14.0 Gm. Bacto dextrose proteose No. 3 agar
15.3 Gm. Bacto nutrient agar
3.2 Gm. soluble starch

Dissolve above in 1,000 c.c. of water and autoclave. Bring the medium to a temperature of 90° C. and add 5 per cent of rabbit or human blood. Dispense sterilely into 6-inch test tubes and slant.

As suggested by Zinsser,[1] we found that the culturing of fairly large quantities of spinal fluid was necessary to obtain satisfactory results. At the same time, it was noticed that organisms from less purulent fluids grew more readily than those from fluids with very high cell counts. Obviously, too many neutrophiles carried over on to the medium inhibited the growth to some extent. We therefore made two sets of cultures on purulent fluids. The uncentrifuged spinal fluid was inoculated, one c.c. into a brain heart infusion broth and 0.5 c.c. onto the starch chocolate agar slant, described above. The remaining portion was centrifuged; the supernatant fluid was poured into a tube of broth and a slant was inoculated with the entire sediment. In many cases growth was obtained on both sets of cultures, but in several instances colonies developed only on the medium inoculated with the unsedimented fluid. Our blood culture

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The report on the pathologic studies of autopsy material in this series has been written by the above authors and will be published under the title "Bilateral Adrenal Hemorrhage Associated with Fulminating Meningococcal Septicemia (Waterhouse-Friderichsen Syndrome)." The clinical report is being written by Captains C. M. Dummer and K. L. Cloninger and will be published at an early date.

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method has been the inoculation of 100 c.c. of brain heart infusion broth with 10 c.c. of blood, and in a portion of the cases a poured plate was also made, using 3 c.c. of blood to an enriched nutrient agar base. To counteract the sulfa drug, para-aminobenzoic acid, 50 mg. per liter of media, was added.

To supply the carbon dioxide requirements of the meningococcus, Thompson[2] suggests 1 Gm. of sodium bicarbonate and 100 c.c. of 3 per cent sulfuric acid for each 2,500 c.c. volume of the incubation jar. In our work we have used an ordinary mason jar. After placing the culture tubes in the jar, the chemicals are added, and after a few seconds to permit the top gases to escape, the top is clamped on. We did not have the opportunity to try carbon dioxide gas and the Novy or similar jar early in our series. However, it is our belief that the combination of carbon dioxide and moisture is the ideal condition, and we have been reluctant to change the above procedure.

It seems worth while to mention here the extreme variation in the size of the meningococcus as seen on stained smears in this series. Organisms isolated from some cases were in the normal range of 1 micron in diameter, but often the organisms from a case seen on the same day would measure anywhere from 1 to 4 microns in diameter. Size variability in a given culture of meningococcus has been recognized as a characteristic of the organism, but the tremendous size of some organisms isolated from a given case has been a constant surprise to us. A large strain seen on direct smear from the spinal fluid has invariably maintained its size on culture, and a like coordination has been seen in cases of the smaller strains.

We have also noticed a wide variation in colony size in different isolations, the majority measuring 1 mm. to 2 mm. in diameter. However, in some instances the colonies have measured more than twice that size, making it difficult to recognize them as meningococcus colonies. No attempt has been made to coordinate size of organism with size of colony produced.

In the 182 cases studied (Table I), we obtained positive cultures in 167 cases or 91.76 per cent. In four cases, not counted in this series, organisms other than the meningococcus were isolated from the spinal fluid. In two of these cases the pneumococcus was isolated from both the spinal fluid and the blood. In the other two cases, *Micrococcus tetragenus* and streptococcus, gamma type, were found in the spinal fluid, and cultures of the blood were negative.

We have considered all purulent fluids, showing no organisms in the sediment, and negative cultures as probable meningococcus fluids and included these as missed cases from the laboratory standpoint. Fifteen cases (Table I), or a total of 8.24 per cent, belong in this group. These patients were charted as meningitis of undetermined etiology, but coming during a meningococcus epidemic, and due to the fact that other pyogenic organisms with a lesser tendency to autolyze were not found, it seems reasonable to suppose that the meningococcus was probably the causative agent.

A comparison of the culture results in spinal fluid and blood shows much greater success in isolating the organism from the spinal fluid than from the blood, the former being positive in 140 cases while the latter was positive in only 85. We can offer no definite explanations for this, since the two fluids were drawn within a few minutes of each other in practically all cases. However, we do believe that positive blood findings would have been materially increased

if repeat cultures at 24-hour intervals could have been made. This procedure was not followed routinely because of the fact that a high level of sulfadiazine was maintained in these patients. It is interesting to note that percentages of positive blood cultures increased late in the series without change in methods. This brings up the question of modes of invasion and whether or not we have in some cases a meningitis without a meningococcemia.

Twenty cases with a variable rash and a leucocytosis are referred to (Table I). Spinal taps revealed clear, colorless fluids with no increase in cells. Other constituents, such as globulin and sugar, showed no change, and cultures of both the spinal fluid and blood were negative. There is, of course, a possibility that some of these cases were meningococcus septicemias. Zinsser[1] advises a serious consideration of the diagnosis of meningococcus septicemia whenever a prolonged case of fever with leucocytosis and a slight variable rash comes under observation, but due to an absolute lack of laboratory evidence, we have not included them in this discussion.

TABLE I

SUMMARY OF 182 CASES STUDIED

CASES WITH POSITIVE LABORATORY FINDINGS		
	NO. CASES	PER CENT TOTAL
N. intracellularis		
C.S.F. Pos., Blood Neg.	80	43.96
C.S.F. Pos., Blood Pos.	60	32.97
C.S.F. Neg., Blood Pos.	25	13.73
C.S.F. Neg., Blood Neg., Petechiae Pos.	2	1.10
Total	167	91.76
CASES WITH NEGATIVE LABORATORY FINDINGS		
	NO. CASES	PER CENT TOTAL
Clinically meningitis of undetermined etiology	15	8.24
Total	182	100.00
Cases with purpura and high WBC, but no other positive findings (not included in series)	20	

TABLE II

CASES WITH CLEAR C.S.F.; POSITIVE ON CULTURE

NO. CASES	CELL COUNT	APPEARANCE	SMEAR	SUGAR	GLOBULIN	CULTURE
10	0- 5	Clear	-*	Normal	Normal	+
4	6-10	Clear	-†	Normal	Normal	+
2	11-15	Clear	-†	Normal	Normal	+
4	16-20	Clear	-†	Normal	Normal	+
5	21-50	Clear	-	Normal	Normal	+

*Four cases in this group showed gram-negative extracellular diplococci in the sediment.
†One case in this group showed gram-negative extracellular diplococci in the sediment.

EARLY MENINGITIS CASES WITH CLEAR SPINAL FLUIDS (TABLE II)

Of unusual interest to us has been the number of meningitis cases from which clear, colorless spinal fluids with normal cell counts and normal chemical constituents were obtained. These fluids gave no sediment on centrifugation, and smears made from the small amount of fluid adherent to the walls of the tube after draining, when stained, showed only an occasional leucocyte. In seven cases, gram-negative extracellular diplococci were found, and in two cases, we were able to demonstrate one or two intracellular organisms, but all others resembled perfectly normal fluids. On culture, all 25 of these cases

showed gram-negative diplococci which on typing proved to be a true meningococcus. Elliott[3] reports such a case with a cell count of 11 polymorphonuclear leucocytes, and other cases have been reported, but this group comprising approximately 14 per cent of our total meningococcus cases would seem to indicate one of two things: either the lumbar puncture was done very early, before the cellular response to the infection, or there was a temporary sacculation in parts of the subarachnoid space. In only three of these cases was a later puncture done, and only one of these showed any material elevation in the leucocyte count.

THE RAPID DIAGNOSIS OF MENINGOCOCCEMIA FROM SMEARS AND CULTURES OF PURPURIC LESIONS

In 1915, Coles[4] reported the finding of meningococci on smears made from peripheral blood. While counting 2,000 leucocytes, "10 were found to contain typical meningococci." His blood films were stained by Giemsa's method, and his conclusion that the organisms observed were meningococci was based solely on their morphologic similarity to gonococci. In 1916, Netter and Salanier[5] demonstrated the presence of meningococci in the purpuric lesions of two cases of meningococcus bacteremia, and Benda[6] was successful in demonstrating the organisms in sections made from the purpuric lesions. Benda, however, was able to detect them in only one case out of five. Credit must be given to Drigalski[7] for having been the first to demonstrate meningococci in the herpetic vesicles associated with meningococcal infections. In Drigalski's case, a blister-like lesion was apparently produced artificially on the ear lobule by rubbing with green soap and alcohol and making smears and cultures from the extravasated serum. He successfully cultured this material, but failed to obtain viable organisms on subculture. Late in 1916, Netter, Salanier, and Wolfrom[8] found meningococci in smears from purpura in a third case, and in 1917, Netter, Salanier, and Blanchier[9] reported two additional cases. In one, positive smears were obtained, and in another they were also successful in obtaining typical colonies on cultivation. Muir in 1919 reported two cases of meningococcic infection with autopsy findings. In both cases smears from petechiae after death showed gram-negative intracellular diplococci, and in one case he grew meningococci from the petechiae and venous blood after death. Reference for bibliography: Muir, R. J.: Note on the Presence of Meningococci in the Skin Petechiae in Cerebrospinal Fever, J. Royal Army Med. Corps **33**: 404, 1919.

Middleton and Duane[10] in 1929 reported an ordinary cover slip smear of peripheral blood revealed a large number of intracellular and extracellular diplococci, resembling meningococci, and in 1931 McLean and Caffey[11] were able to demonstrate meningococci in smears from the purpura in fifteen out of eighteen cases. This report of positive findings in 83 per cent of cases was by far the most important work up to that time, and it now seems surprising that more work was not done in subsequent outbreaks. Nine years later Moss and Schenken[14] observed intracellular diplococci in the granulocytes of ante-mortem blood in one case and demonstrated gram-negative intracellular diplococci in the direct smears made from purpuric spots post-mortem in a second case. In 1942, Kwedar[12] reported that gram-negative intracellular diplococci were found on a blood smear, which were later proved to be meningococci by blood culture.

Monfort and Mehrling[13] stated that microscopically the cutaneous lesions seen in meningococcic bacteremia are shown to be due to direct involvement of the capillaries by the causative organism. Thompkins, in 1943, reported that positive smears were obtained in 39 of 48 cases (about 80 per cent) from purpuric lesions in meningococcic disease. Reference for bibliography: Thompkins, V. N., M. C., A. U. S.: The Diagnostic Value of Smears From Purpuric Lesions of the Skin in Meningococcic Disease, J. A. M. A. **123**: 31, 1943.

We, like many others, were not appreciative of the tremendous value of the stained smear and culture of blood from the purpuric lesions of meningococcic septicemia until rather late in our series.

RESULTS OF SMEARS AND CULTURES FROM PURPURIC LESIONS

Forty cases are recorded in Table III. Blood culture and spinal fluid examination and culture were done simultaneously with the examination of the material from the purpuric lesion.

In Case 1, the patient was extremely ill and already in coma, and because earlier we had failed to diagnose a similar case before death, we decided to attempt cultures and examine stained smears of blood from the petechiae which covered the chest and extremities of the patient. After cleansing the purpuric area with alcohol, we inserted an ordinary 20 gauge needle at an angle almost parallel with the skin surface into the center of the petechial spot, the main concern being not to go deeply enough to draw peripheral blood. By gentle squeezing, several drops of blood and serum were extravasated, and using a sterile platinum loop, cultures and then smears were made of this material. A gram stain was made immediately, and the presence of numerous gram-negative intracellular diplococci was demonstrated (Fig. 1). At approximately eighteen hours, a typical colony growth appeared on the culture medium. On typing, this proved to be a true meningococcus, Type I, and identical with the organism later isolated from both the spinal fluid and blood.

Except for one slight change in technique, we have followed the above procedure in all patients with a petechial or diffuse macular rash. Noting Salanier's reference to the use of a vaccinostyle to scarify the purpuric patch, we have in recent cases, after making the needle puncture, scratched the skin surface over the purpuric area until a slight oozing of blood was obtained. Very light pressure around the area with the fingers produces sufficient material for culture and several smears, with less likelihood of obtaining peripheral blood. We feel that best results have been had with the petechial type of rash, but positive cultures have been obtained from the diffuse type.

Table III (column 1) shows that in 27 cases we were able to find typical gram-negative intracellular diplococci on the smears, thereby permitting a tentative diagnosis within thirty minutes after the patient was admitted to the hospital. In no case in which the organism was found on the direct smear from the purpura did we fail to culture, identify, and type the organism as meningococcus. This, we think, is an important point, since many of the earlier workers did not go beyond finding the organisms on the stained smears. In column two (Table III), it is shown that cultures from the purpuric lesions were positive in 35, or 87.5 per cent, of the total cases. Eight cases in which we did not find typical organ-

isms on the smear were positive on culture. In these cases suspicious extracellular bodies resembling single cocci were often seen, but nothing was definite enough to report as diagnostic.

TABLE III

SMEARS AND CULTURES FROM 40 CASES WITH PURPURIC LESIONS

	SMEARS OF PURPURA	CULTURES OF PURPURA	OTHER LABORATORY FINDINGS	
			C.S.F. CULTURE	BLOOD CULTURE
Case 1	+	+	-	+
Case 2	-	-	+	-
Case 3	+	+	+	-
Case 4	+	+	-	-
Case 5	-	+	-	-
Case 6	-	-	+	+
Case 7	-	+	+	-
Case 8	-*	+	+	+
Case 9	-	-	+	-
Case 10	-	-	+	-
Case 11	+	+	+	+
Case 12	+	+	-	+
Case 13	+	+	+	+
Case 14	+	+	+	+
Case 15	+	+	+	+
Case 16	+	+	+	+
Case 17	+	+	+	+
Case 18	+	+	+	+
Case 19	+	+	+	+
Case 20	-	+	+	+
Case 21	+	+	+	+
Case 22	+	+	+	+
Case 23	+	+	+	+
Case 24†	+	+	+	+
Case 25	-	+	-	+
Case 26	+	+	+	+
Case 27	+	+	+	-
Case 28	+	+	-	+
Case 29	+	+	+	+
Case 30	+	+	+	+
Case 31	+	+	+	+
Case 32	-	+	+	+
Case 33	-	+	+	+
Case 34	-	-	+	-
Case 35	+	+	+	+
Case 36	+	+	+	+
Case 37	+	+	+	-
Case 38	+	+	-	+
Case 39	+	+	+	+
Case 40	-	+	+	+
Per Cent Pos.	67.5	87.5	82.2	75

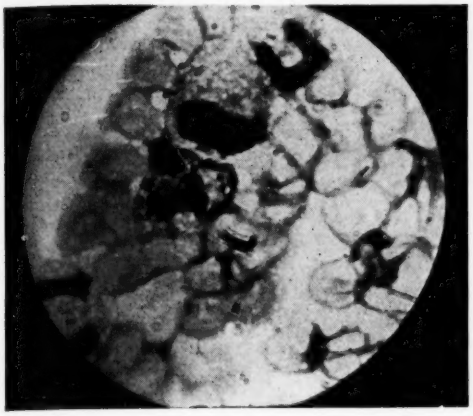
*Slides in this case suspicious but not diagnostic.

†Meningococci were isolated from aspirated knee fluid in this case.

We have isolated the meningococcus from purpura in four additional cases since this paper was written.

The experience of Coles,[4] Middleton,[10] and Kwedar[12] in finding the organisms in ordinary smears of the peripheral blood should be kept in mind, but their cases were unquestionably overwhelming infections, and our experience has not shown it to be a dependable method of diagnosis. In several cases after finding meningococci in smears from the purpuric lesions, we have diligently searched for organisms on ordinary smears of the peripheral blood, but without success.

A comparative analysis of complete findings, including spinal fluid and blood cultures, shows that in 40 cases the cultures of the lesions were positive in 35, while the blood cultures were positive in only 30 cases. It is interesting

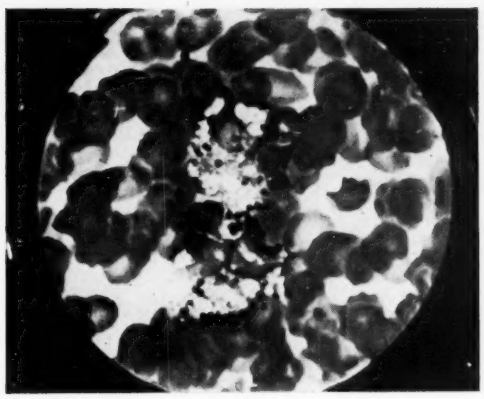


A.

B.

C.

D.



E.

Fig. 1.—Examples of smears from purpuric lesions. *A* shows several pairs of intracellular meningococci (Case 1, Table III), Gram's stain; magnification ×600. *B* shows intracellular meningococci (Case 16, Table III), Gram's stain; magnification ×900. *C* shows numerous meningococci in a disintegrating polymorphonuclear leucocyte (Case 36, Table III). Gram's stain; magnification ×1200. *D* shows a cluster of extracellular meningococci (Case 36, Table III). The erythrocytes have been destroyed by fixing. (Magnification ×1200.) *E* shows two pus cells with large numbers of meningococci (Case 37, Table III), Wright's stain; magnification ×600.

to note that the percentage of positive findings in the cultures of the purpura is higher than the cultures of either the blood or spinal fluid. However, in arriving at comparative values of diagnostic methods, it is important to remember that many cases of meningococcic septicemia with purpura do not develop into spinal meningitis, and in these cases the evidence presented weighs heavily in favor of smears and culture of material from the purpuric lesions (87.5 per cent positive purpura and 75 per cent positive blood cultures). Table III also shows two cases, 4 and 5, in which the material from the purpuric areas furnished the only positive diagnosis in the case. In Case 4, typical gram-negative intracellular diplococci were found on the smear, and positive cultures were obtained. Diligent search in Case 5 failed to demonstrate organisms on the smears, but cultures showed typical colonies which on typing proved to be meningococci. Cultures of the venous blood and spinal fluid done simultaneously, and as in all other cases prior to initiation of the treatment, were both negative. The technique of making and examining the smears is such that we feel it should in all cases be done by the bacteriologist or pathologist and not entrusted to a laboratory technician.

TABLE IV

TYPES OF MENINGOCOCCI

	NO. CASES C.S.F. OR BLOOD	PER CENT TOTAL	NO. CASES PETECHIAE	PER CENT TOTAL
Type I	153	91.6	34	97.0
Type II	7	4.2	0	0
Type IIa	2	1.2	0	0
Type IV	1	0.6	1	2.9
Did Not Type	4	2.4	0	0
Total Cases	167		35	

TABLE V

SUMMARY OF DEATHS AND MORTALITY RATE

Number of proved meningococcic meningitis and meningococcemia cases	167
Number of deaths in this group	4
Mortality rate (per cent)	2.39
Total number of cases, including 15 of undetermined etiology	182
Total number of deaths	6
Mortality rate (per cent)	3.29

Approximately 75 per cent of meningococcus infections in this hospital have shown some degree of purpura; therefore it seems very likely that an earlier initiation of our present method of examining smears and culturing the extravasated blood and serum from these areas would have afforded a diagnosis in some of the 20 cases charted as purpura of undetermined etiology (Table I).

SPECIFIC TYPES OF MENINGOCOCCI

In Table IV, we have recorded the specific types of meningococci found in our series. It is significant that almost all of the cases are classed as Type I. In 153 cases or 91.6 per cent of the total, the organism isolated from the blood, spinal fluid, or both, proved to be Type I meningococcus. In 4 cases, or 2.4 per cent, we did not type the organisms. Three of these cultures were identified by agglutination with a polyvalent meningococcus horse serum, and we did not

at that time have the antisera to do specific typings. In the fourth case, the organisms agglutinated when tested with the polyvalent serum, but we were unable to obtain a specific type. Of the 35 meningococcus cultures isolated from purpuric lesions, 34 or 97 per cent were Type I.

Cultures of the nasopharynx were done on 3,846 meningitis contacts. The carrier rate averaged from 16.5 per cent to 23 per cent.

In Table V, a summary of deaths in both proved and unproved cases is given. In the 167 diagnosed cases there were only 4 deaths. Two other deaths occurred among the cases classed as of unknown etiology.

SUMMARY

1. Meningococci were demonstrated in smears from the purpuric lesions in 27 or 67.5 per cent of 40 cases of meningococcic infections. On culture, the organisms were isolated in 35 or 87.5 per cent. In two cases, this offered the only bacteriologic diagnosis.

2. Positive spinal fluid cultures were obtained in 25 cases of meningitis showing clear fluids with normal chemical constituents.

3. A culture medium for the isolation of the meningococcus is described.

CONCLUSIONS

Smears and cultures from purpuric lesions in meningococcemia offer a rapid method of diagnosis. Cultures of the purpuric areas proved to be the most satisfactory diagnostic procedure.

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PLASMA CLOT TENSILE STRENGTH

II. The Effect of Some Physical Factors, Anticoagulants and Coagulants*

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THE use of autologous plasma clots for the suture of peripheral nerves has raised many questions concerning the factors which may influence their physical characteristics.[1, 2] In earlier reports,[3, 4] a simple method for measuring the tensile strength† of plasma clots was described, and the tensile strength was shown to be roughly proportional to the fibrinogen content of the plasma from which they were formed. This report concerns the results of experiments designed to evaluate the influence of other physical and chemical factors on the tensile strength of plasma clots.

MATERIALS AND METHODS

Human venous blood was used in these experiments. It was withdrawn into a syringe coated with mineral oil. Those samples which were used to prepare unmodified plasma (obtained from blood to which no anticoagulant is added) were transferred to paraffin-lined test tubes packed in ice and then centrifuged in 250 c.c. metal cups filled with ice. Those portions of blood which were used for studies of plasma prepared with anticoagulants were placed in tubes containing measured amounts of the anticoagulant. After centrifuging at approximately 2500 revolutions per minute for five minutes, the plasma was pipetted into test tubes of approximately 8 mm. internal diameter (50 sq. mm. cross section) and allowed to clot either spontaneously or upon the addition of the specified reagents. The tests of tensile strength were carried out on clots which were allowed to stand for a minimum time interval of one hour in the water bath after complete coagulation had occurred. Tensile strength determinations were done in at least triplicate on the clots prepared from each sample of plasma.

The technique used for measuring the tensile strength of plasma clots was essentially that previously described.[3] Careful examination of the results of measurements of the tensile strength of numerous clots prepared from the same plasma indicated that the scatter was so large that approximately 30 clots were necessary to establish a significant mean value. In an attempt to obtain closer agreement between repeated determinations on clots prepared from the same

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†The term tensile strength as used in this paper refers merely to the load at which the clot breaks under the specified conditions of preparation and testing.

material, the procedure followed in preparing and testing the clots was systematically modified to eliminate various suspected sources of error. Since the tensile strength of plasma clots was found to increase when more fluid was expressed, this effect was standardized by transferring the clot to a watch glass, where the excess fluid was allowed to drain off. The clot was touched repeatedly against clean filter paper until a partly dry spot remained. It was then suspended between the jaws of a rubber-sleeved Allis clamp fixed to a rigid support. Injury by rough handling in changing weights was diminished by suspending a paper cup by a silk thread from the lower clip attached to the clot and slowly running in water from a burette with an S-shaped delivery tube. The tensile strength of the clot was taken as the sum of the weights of the cup and clip (10 grams) and the volume of water in cubic centimeters at which the clot broke. Tugging or torsion on the clot during the addition of weights was further reduced by slightly deflecting the string from the lower clamp to the paper cup over a ball-bearing pulley so that the occasional twisting of the cup was not transmitted. Since the range of variation in the results was not decreased by these apparent improvements in technique, the variability was considered to be a property of the clots themselves rather than an artifact of the method of testing. The modifications in the technique of measurement, however, were retained because of their convenience. Since averages of groups of 30 or more clots were necessary to obtain significant mean values, it was not practicable to obtain large enough samples of blood from individual subjects to provide sufficient plasma to permit the performance of each complete experiment on a single specimen. It was therefore necessary to have a method of calculation which would avoid undue weighting of the average by determinations on samples of plasma yielding exceptionally strong clots. This was achieved by reducing the absolute values of the tensile strength of plasma clots to relative units in a scale on which the average tensile strength of the unmodified clots from each plasma was fixed as 100. Such a relative tensile strength (RTS) was obtained by dividing 100 times the absolute tensile strength of each clot by the average tensile strength of the unmodified plasma clots prepared from the same blood specimen. In tabulating the results of each experiment, the mean RTS was calculated for all the clots which were prepared and tested under the same conditions. The standard deviation and standard error were computed in the manner suggested by Pearl.[5]

EXPERIMENTS

I. *The Effect of the Physical Conditions of Coagulation on the Tensile Strength of Plasma Clots.*—

A. Size of Tube.

Although the factors and mechanisms determining the tensile strength of plasma clots are not entirely understood, an elementary analysis of the physical system involved suggests at once that the tensile strength should be proportional to the internal cross-sectional area of the tube in which the clot is formed. This phenomenon, assumed without proof by Kristenson,[6] was studied by comparing series of unmodified clots prepared in tubes of three different diameters. For each specimen of plasma, the average strength of the clots formed in tubes of

8 mm. diameter was used as a basis for calculating the RTS of the clots formed from the same plasma in tubes of different diameter. The results of these experiments are shown in Table I, from which it is clear that the mean RTS per square mm. of clot is constant within limits of experimental error when the area of the tube varies through a range of 300 per cent.

B. Temperature.

To determine whether variation in temperature during incubation and measurement could account for some of the observed variations in the tensile strength of clots prepared from the same sample of plasma, a series of clots were prepared and measured in a constant temperature room at 37° C. For this purpose a single specimen of sodium citrate plasma was used, and the clots formed by the addition of dry calcium chloride. All glassware, reagents, and plasma were brought to 37° C. before coagulation was begun and kept at that temperature throughout the entire experiment. The tensile strength of these clots showed the same amount of variation as clots prepared and tested by the usual method.

TABLE I

THE EFFECT OF THE CROSS-SECTIONAL AREA OF THE TUBE IN WHICH PLASMA CLOTS ARE FORMED ON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	INTERNAL DIAMETER OF TUBE	CROSS-SECTIONAL AREA OF TUBE	MEAN RTS	MEAN RTS PER SQUARE MILLIMETER	STANDARD ERROR OF MEAN RTS PER SQUARE MILLIMETER
39	8.0 mm.	50 mm.^{2}	100	2.00	0.07
37	10.5 mm.	87 mm.^{2}	192	2.21	0.11
29	14.0 mm.	154 mm.^{2}	284	1.84	0.11

TABLE II

THE EFFECT OF THE TEMPERATURE AT WHICH PLASMA CLOTS ARE FORMED ON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	TEMPERATURE ° C.	RTS	STANDARD ERROR OF RTS
33	37	100	3.1
34	20	86	3.8
63	37	100	3.2
62	10	67	3.2

To study the effect of slowing coagulation by lowering the temperature, a series of experiments was carried out on unmodified plasma. The RTS of clots formed at 10° C. and 20° C. was computed by taking the average tensile strength of clots formed at 37° C. as 100. At the lower temperatures, the rate of coagulation was greatly retarded and was found to vary markedly from tube to tube. At 37° C. coagulation was usually complete in 20 to 30 minutes, at 20° C. it usually required 2 to 3 hours and at 10° C., 20 to 24 hours. In general, the clots were allowed to stand for several hours after coagulation appeared complete, but because of the uncertainty as to whether the process was complete, the expressed serum was incubated at 37° C. and the determination discarded if further coagulation occurred. The results of these experiments shown in Table II indicate a definite reduction in RTS when the clots are slowly formed at the lower temperatures.

C. Nature of Surface of Tube in Which Coagulation Occurs.

It is well known that the beginning of coagulation in shed blood is markedly delayed when the blood is collected in a paraffin-lined vessel,[7] and this phenomenon has been applied in the preparation of unmodified plasma clots for nerve suture[1, 2] and tensile strength studies.[3, 4] To evaluate the effect of the surface of tube in which the clots are formed on their tensile strength, a series of unmodified plasma clots was prepared in tubes whose inner surfaces were coated with thin layers of paraffin, latex, and colloidal graphite (aquadag).

It was noted in these experiments as in most of the other studies on unmodified plasma, that the coagulation time varied greatly from clot to clot in each series prepared under the same conditions from the same plasma. In general, however, the clots in rubber or latex-lined tubes formed somewhat more slowly and those in the aquadag-lined tubes more rapidly than those prepared in plain glass tubes. The relative tensile strength of these clots was computed by taking the average value of the tensile strength of the clots formed from each plasma in plain glass tubes as 100.

The results of these experiments are shown in Table III. It is clear that within the range of experimental error, the nature of the surface of the tubes in which the clots were formed had little influence on their tensile strength.

TABLE III

THE EFFECT OF THE LINING OF THE TUBE IN WHICH PLASMA CLOTS ARE FORMED UPON THEIR TENSILE STRENGTH

NUMBER OF CLOTS TESTED	LINING OF TUBE	MEAN RTS	STANDARD ERROR
50	Glass	100	2.8
40	Latex	117	6.1
32	Paraffin	102	6.9
32	Graphite (aquadag)	84	6.2

D. Time.

The effect of the period of incubation upon the tensile strength of plasma clots may be important in relation to their suitability as nerve suture material, since if the clots rapidly deteriorate before fibrous tissue replacement has occurred, then the nerve ends may separate. In an earlier report, it was concluded that unmodified plasma clots formed in glass tubes reach their maximum strength about twenty minutes after the beginning of coagulation. In these experiments the beginning of coagulation was determined by tipping the tube at short intervals, noting the time when the plasma failed to flow (tip test). Further study has shown that if the tubes are not agitated, and the end point of coagulation is determined by the appearance of turbidity, there is much variation in the coagulation time among different samples of the same plasma and also in the time required for complete coagulation. To investigate this phenomenon further, 22 one c.c. samples of the same chilled unmodified plasma were placed in a water bath at 37° C. At short intervals these tubes were inspected without agitation, and those in which coagulation appeared to be complete as judged by the attainment of maximum turbidity were removed and tested for tensile strength. It was found that among the tubes in which opacity appeared to be maximal, most of the clots which were examined within the first

forty minutes showed evidence of incomplete coagulation either by sacklike forms of the clots or by the spontaneous coagulation of the expressed fluid. Among those clots which did not show the signs of incomplete coagulation, no significant difference in the tensile strength was observed between those clots incubated twenty minutes and those incubated for longer intervals up to one hundred and forty minutes.

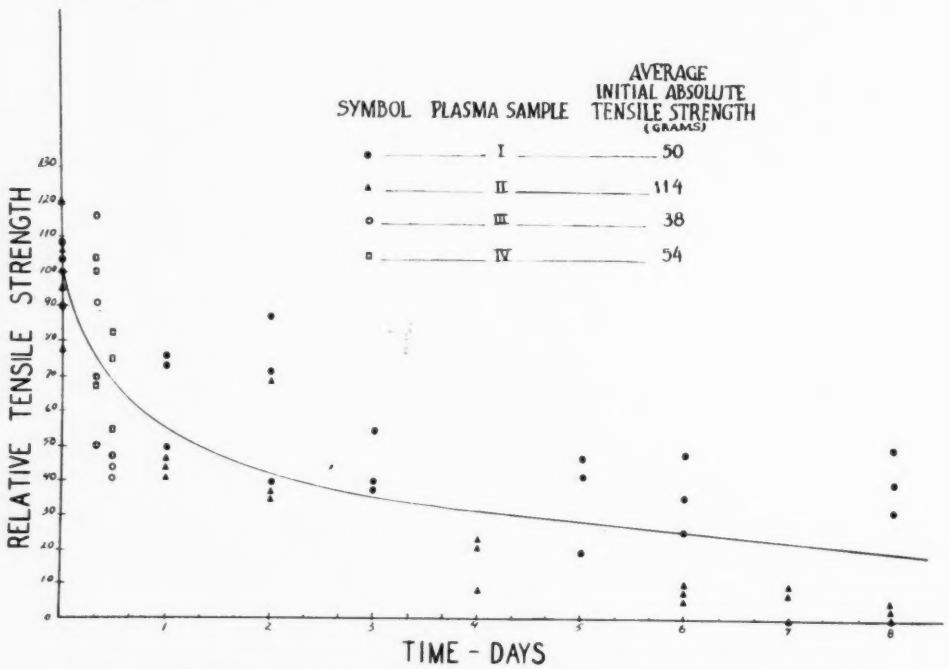

FIG. 1 - CHANGE IN TENSILE STRENGTH OF PLASMA CLOTS WITH TIME.

To study the change in tensile strength of plasma clots on longer standing, it was necessary to take precautions against bacterial contamination and against the loss of water and dissolved gases by evaporation from the surface. To do this, clots were prepared from unmodified plasma obtained under aseptic conditions and placed in sterile test tubes into which a sterile cotton plug soaked with melted paraffin was pushed to within about 1 cm. of the surface of the plasma. The tubes were then closed with sterile corks and sealed with paraffin. On several batches of plasma handled in this way and incubated at 37° C., triplicate tensile strength determinations were done at the beginning of the experiment and at various time intervals thereafter. The RTS was calculated using the average tensile strength for the clots from each plasma at the beginning of the experiment as 100. The results are shown graphically in Fig. 1. In spite of the wide scatter of individual determinations, it is clear that the tensile strength declines rapidly within the first 24 hours reaching a value 50 to 60 per cent of its initial strength and then falling more slowly to about 20 per cent of its initial value at the end of eight days. Partial lysis was noted in some of the clots by the end of the fourth day.

II. *The Effect of Tissue Extracts on the Tensile Strength of Plasma Clots.*—Extracts of mammalian tissue contain substances, probably lipoprotein in nature, which accelerate the coagulation of fresh blood or plasma. The exact mechanism of their action is uncertain, but it is known that in the presence of calcium ions, they accelerate the conversion of prothrombin to thrombin which in turn catalyzes the conversion of fibrinogen to fibrin.[7]

Since lung and muscle extract have been added to the unmodified plasma used in some of the studies on plasma clot suture,[2] the effect of these substances on the strength of clots was investigated. Tissue extracts were prepared by thoroughly grinding fresh human, dog, or rabbit lung or muscle in a mortar with clean sand and centrifuging the mixture. The supernatant fluid then was removed and used undiluted, or diluted five and ten times with Ringer's or normal saline solution. In testing these preparations, one drop was added to one c.c. of chilled unmodified plasma. The results of the tensile strength determinations are shown in Table IV.

TABLE IV

THE EFFECT OF MUSCLE AND LUNG EXTRACT ON THE RELATIVE TENSILE STRENGTH OF PLASMA CLOTS*

TYPE OF PLASMA	COAGULATING AGENT	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	None	12	36	100	2.5
Unmodified	Muscle extract	12	51	72	4.8
Unmodified	Muscle extract diluted 1:5	12	51	83	3.6
Unmodified	Muscle extract diluted 1:10	12	41	87	4.9
Unmodified	Lung extract	12	36	34	3.9
Unmodified	Lung extract diluted 1:5	12	36	66	3.9
Unmodified	Lung extract diluted 1:10	12	29	78	6.1

*The data presented in the above table are not strictly comparable to those presented in Tables V and VI, since but one drop of muscle or lung extract was added to the unmodified plasma. Hence the dilution factor in these experiments with muscle and lung extract is negligible.

The muscle and lung extracts were diluted with either Ringer's or normal saline solution.

The coagulation accelerating tissue extracts all tended to weaken the clots produced from unmodified plasma, an effect more marked with lung extract, which produced more acceleration of coagulation than muscle extract. That the effect is not due entirely to the increased coagulation rate is indicated by the finding that the diluted extracts, which had about the same effect on the rate of coagulation, weakened the clots less than the more concentrated ones.

III. *The Effect of Anticoagulants on the Tensile Strength of Plasma Clots.*—

A. Decalcifying Anticoagulants.

The most commonly used anticoagulants are the soluble salts of citric and oxalic acid which prevent coagulation by removing calcium ions from solution. Plasma prepared by centrifuging blood treated with these anticoagulants may be recoagulated by the addition of suitable amounts of soluble ionized calcium salts.

Solutions isotonic with blood plasma were used, since other concentrations would produce a shift of water between the red blood cells and the plasma until equilibrium was re-established.

1. Sodium Citrate.

A 3.8 per cent solution of hydrated sodium citrate is isotonic with blood plasma. Preliminary trials of several different blood samples containing varying amounts of sodium citrate indicated that one part of citrate to 19 parts of whole blood is a safe anticoagulant for most specimens and that one part to 14 of whole blood is safe for all bloods examined. These figures, representing 0.14 per cent to 0.18 per cent anhydrous sodium citrate in the final mixture, are in good agreement with those (0.15 per cent to 0.20 per cent) reported as minimal anticoagulant concentrations in the literature.[8]

The minimal amount of calcium chloride necessary to coagulate citrated plasma may be calculated from the theoretical studies of Ransemeier and McLean,[9] but since more than minimal amounts are probably optimal, the data of Nygaard[10] were used as a starting point. A rough calculation from these data suggests that 0.1 c.c. of a 0.5 per cent to 2.5 per cent solution of $CaCl_2 \cdot 2H_2O$ should act as a convenient and effective coagulant.

Citrated plasma was prepared from the blood of each of two healthy normal adults using one part of a 3.8 per cent sodium citrate solution to 19 parts of blood. Nine-tenths c.c. portion of this plasma was mixed in each tube with 0.1 c.c. of a solution of calcium chloride of 0.5, 1.0, 1.5, 2.0, or 2.5 per cent. At least three clots were prepared at each calcium chloride concentration. The tensile strength was determined on these clots and on unmodified clots prepared from each sample of plasma. The RTS was calculated. As was expected from Nygaard's observations, no significant difference was noted between the clots prepared with the different amounts of calcium chloride.

A similar experiment was carried out using a fixed amount of calcium chloride and varying the concentration of the sodium citrate solution from 5 volumes to 11 volumes per cent. Here the larger concentration of citrate seemed to give somewhat weaker clots, but the difference was not large compared with the difference between triplicates of the same sample.

On the basis of these two experiments, it was considered that slight differences in citrate concentration such as might occur as a result of unusually high or low hematocrit values would be unlikely to cause significant differences in plasma clot tensile strength, and that small differences in the proportion of calcium chloride to sodium citrate would also have relatively little effect.

2. Potassium and Ammonium Oxalate.

A 1.6 per cent solution of a mixture of 4 parts of dry potassium oxalate and 6 parts of dry ammonium oxalate is isotonic with blood plasma. Preliminary trials of this solution with different proportions of whole blood indicated that one part of the oxalate solution is a safe anticoagulant for 19 parts of blood. Calculations show that this figure corresponds closely to Scott and Chamberlain's[11] value which represents four times the amount necessary to combine with the plasma calcium. Experiments with oxalate solution similar to those carried out with the citrate solution gave analogous results. When, however, 1 part of oxalate solution was added to 9 parts of blood, clotting occurred although the clots were very weak.

To study in detail the RTS of clots prepared from recalcified plasma, unmodified, citrated, and oxalated plasmas were prepared from the blood of each of ten subjects. Isotonic potassium and ammonium oxalate solution was used in the proportion of one part to 19 parts of blood. Isotonic sodium citrate solution was used in the proportions of one part to 14 parts and one part to 19 parts of blood. One-tenth c.c. of 2 per cent calcium chloride solution was added to 0.9 c.c. of chilled plasma containing the anticoagulant and mixed by inverting the test tube three times against a clean cork. The RTS of the resulting clots were determined.

To evaluate the effect of the dilution of the plasma by the added reagents, a series of determinations of RTS was done on additional groups of clots prepared from unmodified plasma to which isotonic sodium chloride solution was added in the proportion of 10 per cent and 20 per cent of the final volume. As a further control, the RTS was determined on a series of clots prepared from unmodified plasma to which the citrate and oxalate salts had been added in dried form in the same proportions in which they would occur in plasma obtained from blood to which one part of isotonic anticoagulant solution had been added for each 14 parts of blood. These plasmas were coagulated by adding 1 c.c. portions to test tubes containing the calcium chloride dried from 0.1 c.c. of a 2 per cent solution. The results of all the studies on decalcifying anticoagulants and dilution controls are shown in Table V.

When an isotonic solution of an anticoagulant salt is added to whole blood, the plasma is diluted by it. It may be easily calculated* that clots prepared from 0.9 c.c. of such plasma and 0.1 c.c. of calcium chloride solution contain fibrinogen, and other solids present in only about 0.8 c.c. of original blood plasma and their mean RTS may, therefore, be expected to be about 20 per cent less than that of clots prepared from unmodified plasma.[4] In Table V it is seen that the clots prepared from unmodified plasma diluted with 20 per cent of saline solution averaged 27 per cent weaker, while the citrated clots were 21 per cent weaker, and the oxalated clots 30 per cent weaker than clots prepared from unmodified plasma. The standard errors of these results are large enough so that it cannot be concluded that any of these values is significantly different from the 20 per cent weakening effect expected as the result of dilution alone.

For citrated plasma clots, this interpretation is further strengthened by the finding that unmodified plasma stabilized by the addition of dry sodium citrate and coagulated by the addition of dry calcium chloride gave clots whose mean RTS was not significantly different from 100, the mean RTS of the unmodified clots.

On the other hand, the clots prepared from unmodified plasma treated with dry potassium ammonium oxalate and dry calcium chloride had a mean RTS 14 per cent less than that of the unmodified clots. Since the standard

*The volume of original plasma in the final 1 c.c. clot is $\frac{0.9 \text{ c.c.} \times \text{volume of plasma}}{\text{volume of plasma} + \text{volume of anticoagulant.}}$

The volume of plasma equals volume of blood × (1 − hematocrit). With a hematocrit of 0.45:

1 c.c. of anticoagulant to 14 c.c. of blood gives: $\frac{0.9 \text{ c.c.} \times (14 \times 0.55)}{(14 \times 0.55) + 1} = 0.80$ c.c.

1 c.c. of anticoagulant to 19 c.c. of blood gives: $\frac{0.9 \text{ c.c.} \times (19 \times 0.55)}{(19 \times 0.55) + 1} = 0.82$ c.c.

error is 5.1 per cent, this may be considered as representing a small but definite weakening effect due to the oxalate, possibly caused by the presence of crystals of calcium oxalate among the fibers of the clot.

B. The Use of Heparin as an Anticoagulant.

The anticoagulant action of heparin has recently been reviewed by Quick,[7] who summarizes as follows: "first, it prevents the liberation of thromboplastin from platelets; second, it prevents, with the aid of a plasma co-factor, the conversion of prothrombin into thrombin; and third, it forms with serum albumin a strong antithrombin." Thus, heparin, by acting as an inhibitor of coagulation at three successive stages of the clotting process, is admirably suited for the role for which it has often been suggested, but never conclusively proved—that of the normal physiologic anticoagulant.[7, 12] For our purposes, it has the added advantage of being extremely potent in small amounts.

TABLE V

THE EFFECT OF RECALCIFICATION OF CITRATED AND OXALATED PLASMA AND OF DILUTION WITH SALINE SOLUTION ON THE RELATIVE TENSILE STRENGTH OF CLOTS

TYPE OF PLASMA	RECALCIFIED OR DILUTED WITH	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	Nothing	10	46	100	2.8
Sodium citrate (1:20)	0.1 c.c. of 2 per cent calcium chloride	8	31	73	5.6
Sodium citrate (1:15)	0.1 c.c. of 2 per cent calcium chloride	10	39	83	6.6
Sodium citrate (1:15 or 1:20)	0.1 c.c. of 2 per cent calcium chloride	10	70	79	4.3
Ammonium and potassium oxalate solution (1:20)	0.1 c.c. of 2 per cent calcium chloride	10	40	70	4.3
Unmodified	Nothing	16	87	100	2.9
Unmodified	0.1 c.c. of isotonic sodium chloride	11	66	89	4.4
Unmodified	0.2 c.c. of isotonic sodium chloride	13	79	73	3.3
Unmodified	Nothing	5	35	100	4.4
Unmodified dry sodium citrate	2 mg. dry calcium chloride	5	40	97	5.3
Unmodified dry ammonium and potassium oxalate	2 mg. dry calcium chloride	5	38	86	5.1

Preliminary tests of a 1 per cent solution of the sodium salt of heparin (Liquaemin: Roche) indicated that while 0.01 mg./c.c. of blood is adequate to prevent coagulation of some specimens, 0.05 mg./c.c. is necessary for a safe anticoagulant effect, and this amount was used in all subsequent experiments.

To coagulate heparinized plasma, one must either supply an excess of thrombin, or else neutralize the heparin so that the normal coagulation mechanism can operate. Both methods were studied.

1. Coagulation of Heparinized Plasma by the Addition of Rabbit "Clotting Globulin."

"Clotting Globulin" (Lederle)* is derived from rabbit plasma, and it has been demonstrated to have the properties of thrombin.[13, 14] It is supplied in a 10 per cent solution which contains a preservative.

*Kindly supplied by the Lederle Laboratories.

In exploratory experiments with varying amounts of globulin as the coagulating agent for heparinized plasma, it was found that 0.1 c.c. of the stock 10 per cent solution added to 0.9 c.c. of heparinized plasma or 0.05 c.c. of the solution mixed with 0.95 c.c. of plasma gave clots which were up to 50 per cent weaker than the average clot prepared from unmodified plasma. One-tenth c.c. of a 2 per cent solution to 0.9 c.c. of plasma yielded clots from 20 per cent to 80 per cent weaker than unmodified plasma clots, and 0.05 c.c. of 2 per cent solution added to 0.95 c.c. of plasma resulted in clots which were so weak and friable that their strength could not be measured.

Unmodified plasma and heparinized plasma (0.05 c.c. of 1 per cent solution of liquaemin to 10 c.c. of blood) were prepared from blood taken from each of ten patients. Nine-tenths c.c. of heparinized plasma was coagulated with 0.05 c.c. or 0.1 c.c. of 10 per cent "Clotting Globulin" solution. Unmodified clots were prepared as usual. The tensile strengths were measured and the RTS calculated. The results are shown in Table VI.

TABLE VI

THE EFFECT OF RABBIT "CLOTTING GLOBULIN" AND PROTAMINE SULFATE ON THE RELATIVE TENSILE STRENGTH OF HEPARINIZED PLASMA CLOTS*

TYPE OF PLASMA	COAGULATING AGENT	NUMBER OF SAMPLES OF PLASMA	NUMBER OF CLOTS TESTED	MEAN RTS	STANDARD ERROR
Unmodified	None	10	46	100	2.8
Heparinized	"Clotting Globulin" 0.05 c.c.	3	12	60	12.6
Heparinized	"Clotting Globulin" 0.1 c.c.	7	28	57	6.2
Heparinized	"Clotting Globulin" 0.05 c.c. or 0.1 c.c.	10	40	58	6.3
Unmodified	None	10	54	100	4.0
Heparinized	Protamine sulfate 0.1 mg.	10	41	100	3.1
Heparinized	Protamine sulfate 0.125 mg.	10	41	106	6.0
Heparinized	Protamine sulfate 0.15 mg.	5	20	102	6.0
Heparinized	Protamine sulfate 0.1, 0.125 or 0.15 mg.	10	102	101	3.0

*The final volume of all clots was 1 c.c.

2. Coagulation of Heparinized Plasma by Means of Protamine Sulfate.

In attempts to obtain an anticoagulant with prolonged action, Chargaff and Olson[15] precipitated heparin with protamine sulfate but found that its effect was completely neutralized. In further studies they showed that protamine sulfate is capable of neutralizing the anticoagulant effect of heparin both in vitro and in vivo. Protamine sulfate, therefore, seemed a suitable reagent for removing heparin to permit coagulation to occur in a normal way. (Protamine sulfate itself is a weak inhibitor of coagulation[15] rather than a coagulant.)

To determine optimal conditions, varying amounts of protamine sulfate were added in 0.1 c.c. of water to 0.9 c.c. of heparinized plasma in a water bath at 37° C., and coagulation or its absence was noted at intervals. It was found that with 0.09 mg. of protamine sulfate or less, coagulation was markedly delayed or prevented, while with 0.1 mg. or more, coagulation was about as rapid as in unmodified blood.

Unmodified plasma and heparinized plasma were prepared from blood drawn from each of ten patients. Nine-tenths c.c. sample of heparinized plasma was coagulated in quadruplicate with 0.1 c.c. of aqueous solutions con-

taining 0.10, 0.12, and 0.15 mg. of protamine sulfate respectively. The RTS of the clots were determined (Table VI).

The volume and molar concentration of heparin used as an anticoagulant in our experiment was so small that it introduced no significant dilution, but the added coagulating agents produced a slight but definite dilution—5 per cent in the experiment with 0.05 c.c. of "Globulin" solution, 10 per cent in all other experiments. The clots prepared from heparinized plasma coagulated with rabbit "Globulin" had a mean RTS about 40 per cent less than that of the clots prepared from unmodified plasma and must be considered significantly weaker than the latter.

On the other hand, the clots prepared by neutralizing the heparin with protamine sulfate had a mean RTS which was the same as that of the unmodified clots and slightly but significantly higher than the mean RTS of the clots prepared from unmodified plasma diluted with a corresponding volume of saline.

DISCUSSION

Some of the implications of the results of our studies concerning the influence of various factors on the tensile strength of plasma clots are clear. As would be expected, the mean relative tensile strength is proportional to the cross-sectional area of the tube in which the clot is formed, and inversely proportional to the amount of dilution of the plasma by saline solution. These findings are consistent with the previous observation[4] that clots of the same size prepared from different specimens of plasma have tensile strengths roughly proportional to their fibrinogen concentration.

The mean RTS of plasma clots is slightly decreased by the addition of small amounts of muscle extract, and by lowering the temperature from 37° C. to 20° C., while it is slightly increased in latex-lined tubes as compared with glass tubes. Insofar as these compensating factors are concerned, it is likely that the tensile strength of plasma clots in vivo is similar to that observed in vitro. It is significant, however, that the clots in vitro show a definite weakening on standing even when contamination, and evaporation of dissolved gases is prevented.

Some conclusions may be drawn as to the suitability of various types of anticoagulants and coagulants for the preparation of plasma clots for surgical use. The findings on the mean RTS of clots prepared by the recalcification of citrated plasma are consistent with the concept that sodium citrate solution in suitable concentration exercises no effect on the RTS of plasma clots other than that of simple dilution. Since it has been shown that within a fairly wide range the RTS is independent of the volume of citrate solution used as the anticoagulant and the amount of calcium chloride used to coagulate the plasma, and since sterile sodium citrate solution is so readily available wherever surgery is performed, citrated plasma clots would seem to be worthy of further investigation.

The clots prepared by the recalcification of oxalated plasma have a mean RTS slightly but definitely lower than would be predicted as a result of simple dilution. Although this effect is small as compared to the differences between some of the individual clots in the series, the presence of calcium oxalate crystals in the clots and the absence of any theoretical or practical advantage of oxalate over citrate suggest that oxalated plasma is an unsuitable source of clots.

When heparin is used as the anticoagulant, the plasma clots prepared by the addition of the thrombin-like substance from the globulin fraction of rabbit plasma had a mean RTS definitely lower than that expected as a result of dilution alone. Moreover, the fact that they contain relatively large amounts of rabbit protein might make such clots undesirable.

When protamine sulfate solution is used to inactivate the heparin in heparinized plasma and permit the normal coagulation mechanism to operate, the resulting plasma clots have a mean RTS approximately equal to that of unmodified plasma. Since the volume and molar concentration of the heparin-protamine sulfate solutions are so low, the heparin-protamine plasma clots may be considered chemically most similar to unmodified clots and are certainly well worthy of further study in connection with their surgical use.

The fibrin clot in mammalian blood plasma forms when, as a result of the catalytic action of thrombin, the soluble protein fibrinogen changes to the insoluble protein fibrin which then precipitates out of solution.[7] Plasma clots have been found, by microscopic examination, to be composed of interlocking needles,[7] and by x-ray spectrography, each needle has been shown to be built up in a definite crystal structure out of regularly oriented molecules.[16, 17] Thus far, however, no information is available from which it is possible to determine definitely whether the breaking of a clot under tension is the result of the rupture of needles in the fibrin mesh or of the separation of the individual fibers. In the one case the orientation of individual molecules in a fiber, in the other the orientation of fibers in the mesh, would be the important factor in determining the tensile strength of a clot. It is possible that both factors are involved.

Attempts to eliminate the factor of variability when determining tensile strength by modifying the method of preparing and testing the clots were unsuccessful. For this reason the variability may have to be considered as an intrinsic property of the clots. It is a common observation in testing the elastic and mechanical properties of many substances that their stress-strain curves rise smoothly up to the neighborhood of the breaking point where they change their direction sharply. If such a phenomenon occurs in our clots, the measurement of their tensile strength is complicated by changes occurring during the breaking process. Accurate stress-strain curves are necessary to determine how important this type of phenomenon is in causing the variability of our data. On the other hand, the variability in the tensile strengths of plasma clots may also be affected by the complexity of the mechanism of fibrin formation and the conditions of its precipitation.

The rate of thrombin formation may conceivably play an important role. Quick[7] has pointed out that the amount of thrombin required to coagulate plasma in five minutes is only slightly greater than that required to produce the same effect in several hours. Nygaard[10] by means of photoelectric studies on the coagulation of recalcified citrated plasma has shown that the duration of the period of fibrin formation increases when the time for the onset of coagulation is prolonged. This means that relatively minute differences in the rate of conversion of prothrombin to thrombin such as might be caused by minor differences in the amount of incidental injury to platelets in pipetting and manipulation may cause marked differences in the time required for the conversion of

fibrinogen to fibrin, perhaps with corresponding differences in the structure and arrangement of the fibrin strands, and consequently in the tensile strength of the clot.

It is known that the pattern of crystallization of cupric chloride as a result of the drying of solutions mixed with hemolyzed blood varies remarkably with the blood of different individuals in different pathologic states.[18, 19] It would, therefore, not be surprising should the pattern of crytallization of the very much more complex fibrin molecule be found to be even more sensitive to as yet undetermined factors in the blood plasma.

SUMMARY

1. The mean tensile strength of plasma clots is proportional to the cross-sectional area of the tubes in which they are formed.

2. The nature of the surface of the test tube in which clots are formed influences the tensile strength of the plasma clots to a slight extent.

3. The mean relative tensile strength of plasma clots formed at 10° C. and 20° C. is lower than that of clots formed at 37° C.

4. Plasma clots incubated aseptically at 37° C. with precautions against evaporation of fluids and dissolved gases gradually weakened during a testing period of eight days.

5. By a comparison of the tensile strengths of groups of clots prepared from anticoagulant-containing plasmas by adding appropriate amounts of coagulants with the strengths of unmodified plasma clots prepared from the same blood samples, it has been demonstrated that:

A. Clots prepared from unmodified plasma diluted with saline solution are weakened approximately in proportion to the amount of dilution.

B. Clots prepared from unmodified plasma to which muscle and lung extract are added coagulate more rapidly than the controls, but are weakened by more than the dilution factor. Lung extract reduces the tensile strength more than muscle extract.

C. Clots prepared by recalcifying plasma obtained from citrated blood are weakened to an extent roughly proportional to the dilution of the plasma. Clots prepared by adding suitable amounts of the citrate and calcium chloride in dry form to unmodified plasma are as strong as those prepared from unmodified plasma.

D. Clots prepared by recalcifying plasma obtained from oxalated blood are weakened to a slightly greater degree than would be expected on the basis of dilution alone. Plasma to which oxalate and calcium chloride were added in dry form also yields clots which are weaker than their controls.

E. Clots prepared by adding rabbit "Globulin" to the plasma obtained from heparinized blood are weaker than would be expected as a result of dilution.

F. Clots prepared by adding protamine sulfate solution to the plasma obtained from heparinized blood show no weakening as compared to the controls.

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LABORATORY METHODS

GENERAL

APPARATUS FOR CONSTANT RATE INTRAVENOUS INJECTION OF ULTRA SHORT-ACTING ANESTHETICS IN MICE*

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THE apparatus to be described, consisting of a mouse holder and injection machine, was designed to facilitate constant rate intravenous injections in mice of ultra short-acting anesthetics. The mouse holder (Fig. 1) is modified from Burdon's[1] design and consists of a wire and metal cage, hinged at the forward end (*1*) and held down at the rear by a lug (*2*) on the right side. The front is closed by a plate which adjusts lengthwise for different sized mice, and the back is made of another plate soldered to the cage and providing a hole for the tail. The mouse holder is mounted on a paraffined wood block, which is supported by a 7 inch length of ¾ inch threaded iron pipe. Screwed over the pipe are two flanges, one facing upward and attached to the wood block by screws, and the other (*3*) facing downward and resting on the table top. The height of the holder is adjustable by screwing the bottom flange up or down, while rigidity is insured by a washer and nut on the pipe projecting beneath the table. Ordinarily a height is used which will just allow a 150 c.c. beaker beneath the tail of the mouse. It is our experience that immersing the tail for about one-half minute in water at 45° C. gives the best dilatation of tail veins.

The injection machines described in the literature [2-8] are not very satisfactory for the ultra short-acting anesthetics because of the presence of one or more objectionable features; namely, pulsating flow, uncertain control of rate, lack of provision for continuous variation between different rates over a wide speed range, and the difficulty of adaptation of the machine so that large numbers of mice can be easily and quickly injected with small accurately measured volumes.

The present machine (Fig. 1) consists of a source of power, variable speed device, flexible drive, screw, and syringe clamp. An inexpensive rim-drive phonograph turntable, which comes complete with synchronous motor, supplies the power. The turntable (*19*), from which the felt was removed by soaking in hot water, supports a steel wheel (*18*), on the rounded rim of which is cemented a wide rubber band (*16*). The axle (*15*) of this wheel rests in open slot brass bearings (*14, 17*) on each side of the turntable, and these slots are adjustable vertically to permit the use of different sized wheels. A ¾ inch length of speedometer cable (*12*) is sweated into two sleeves, one of which (*13*) receives

*From the Department of Pharmacology of the Vanderbilt University School of Medicine. Received for publication, Sept. 6, 1943.

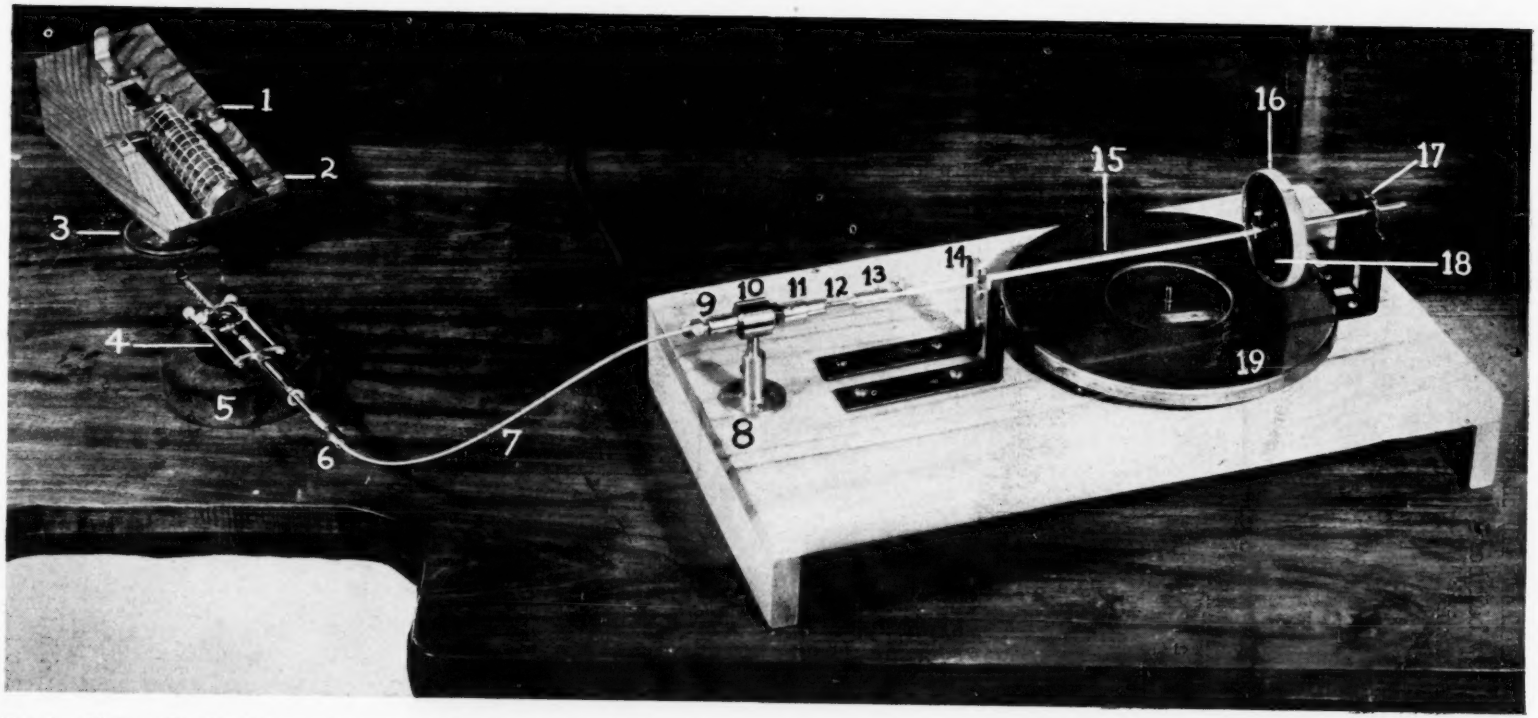

Fig. 1.—NOTE: To simplify construction the handmade ball bearing shown above (*10*) can be replaced by a standard bearing described in the text and illustrated in Fig. 2.

the axle, while the other (*11*) slips over a short steel rod which is snugly fitted inside a double row ball bearing (*10*). This bearing (Fafnir No. 5200), Fig. 2, is held by a setscrew inside a piece of steel pipe, which is counterbored to let the bearing in against a shoulder and tapped beneath for a supporting rod adjustable for height.

In the syringe clamp and screw assembly (Fig. 3), the plunger of a ¼ c.c. syringe is driven by a screw connected to the rod within the ball bearing by a 1 foot length of fairly stiff flexible cable (*7* in Fig. 1), held at each end in chucks (*6*, *9* in Fig. 1). Cable and chucks are Ceneo No. 18812. The screw, 32 threads per inch, runs through a thread and is steadied by a small brass bearing. The syringe is held in a brass clamp which is fastened to the screw mechanism by two knurled nuts. A small spring bears lightly against the shaft of the plunger and helps to hold the latter fixed after adjustment of the dose has been made. Removal of the knurled nuts permits the syringe with its clamp to be detached for cleaning without disturbing the alignment, and with additional clamps, rapid interchange of several syringes containing different solutions is facilitated.

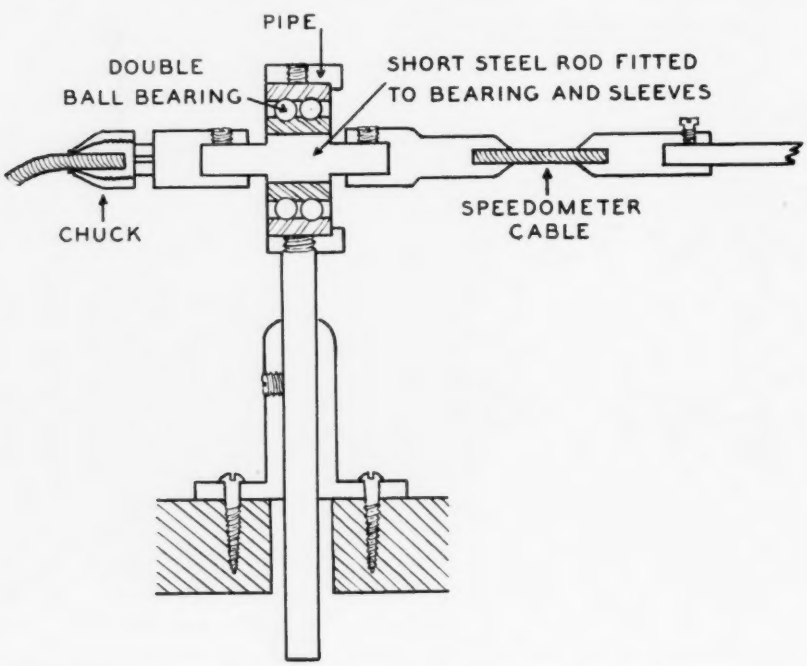

Fig. 2.—Cross section view of ball bearing, support, and drive shaft connections.

The position of the injection machine platform relative to the mouse holder is adjusted so that the flexible cable (*7*) lies in a quarter circle. This allows the right hand to lie under the cable and grasp the syringe from the right, while enough slack is left in the cable so that as the screw revolves it can take the cable forward.

The dose adjustment is made by lifting the axle (*15*) to free the wheel (*18*) from contact with the turntable, running the screw back by hand, and drawing the solution into the syringe past the desired mark. Then the screw is similarly

run forward until the plunger reaches the exact mark, which can be attained within 1 or 2 thousands of a cubic centimeter. During this latter procedure, in order to avoid dosage errors, the syringe clamp and screw assembly (*4*) is held flat. In that position, it is then laid down on a cork ring (*5*), and the injection is made later without allowing the clamp to rotate to the left, since this would cause a premature ejection from the syringe. In practice, this precaution has proved easy to observe.

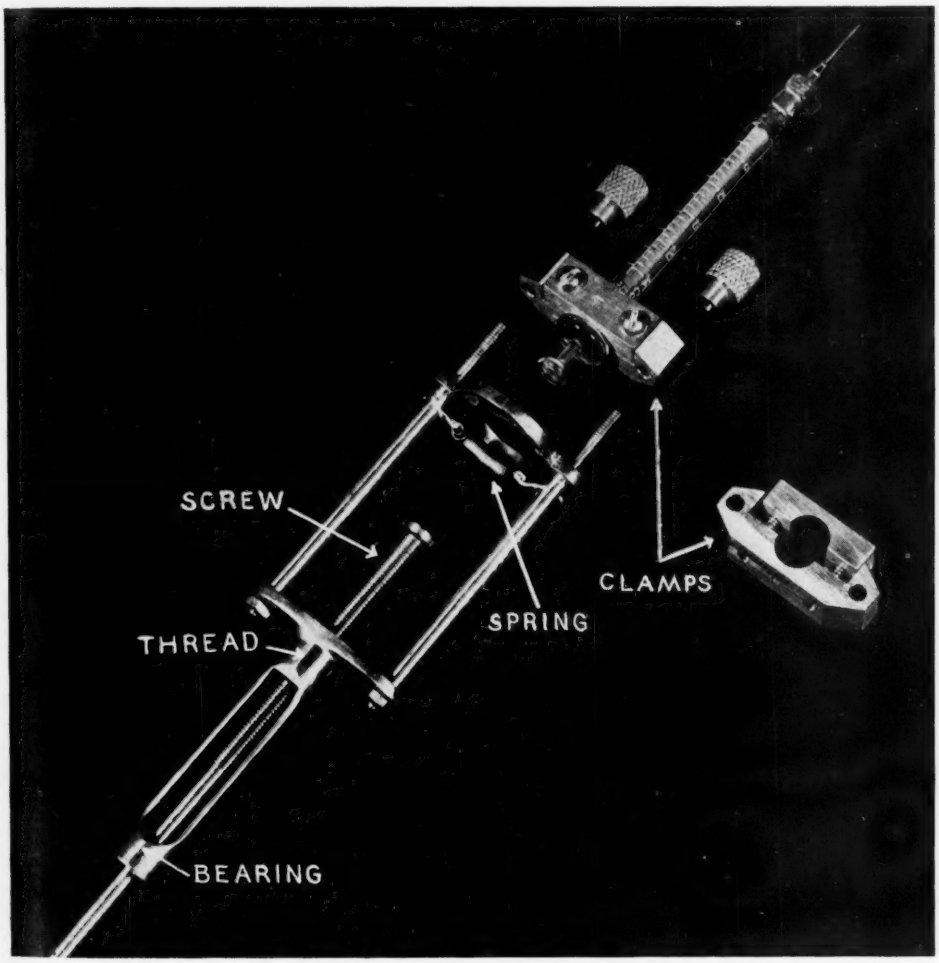

Fig. 3.—Syringe clamp and screw assembly.

When the electric current is turned on by means of a footswitch, the speed of the turntable reaches its maximum in about a second. If this lag should be found objectionable, it could be practically eliminated by mounting a solenoid under the right end of the axle (*15*), and with the turntable running continuously, by starting and stopping the injection through the vertical movement of the solenoid core acting to lower or to raise the wheel (*18*). The speed of the injection can be varied over a range of from about 0.002 to 0.04 c.c. per second, by using two interchangeable wheels (*18*), 1½ inch and 3 inches in diameter, and

varying the wheel position on the turntable. A steel mechanic's rule graduated in 0.5 mm. divisions is quite satisfactory for determining the wheel position. The exact error in rate for rapid injections, lasting only a few seconds, is difficult to determine with a stop watch; but with longer injections, for example, thirty seconds, the error is less than 2 per cent.

At the end of injection, as the plunger seats home, the clamp begins to rotate clockwise in the hand, which insures a full dose and also acts as a signal to step on the footswitch and shut off the motor. There is very little danger of breaking the syringe or plunger, since the flexible components of the drive take up the shock, which if prolonged, merely causes the wheel to skid on the turntable. The only occasion on which a syringe plunger broke was when the syringe was not well aligned with the screw. This alignment can be improved if necessary by shimming with small pieces of paper at the joint where the syringe clamp is attached to the screw mechanism by the knurled nuts. It may be found necessary to grind off the head of some plungers or cement a small piece of cardboard on the head to true it up in relation to the adjoining face of the screw.

This machine has been used in about 2,000 intravenous injections in mice, and presents the following advantages: (a) The dose can be quickly and accurately measured in a power-driven syringe which retains the necessary maneuverability for direct injection of tail veins. (b) The injection proceeds at a smooth and reproducible rate which can be varied continuously over a considerable range. (c) The machine is relatively simple and inexpensive to construct.

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A NEW HEMATOCRIT TUBE

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ITHACA, N. Y.

THE author has recently reviewed a publication on the anemia of flexed-tailed mice by Grüneberg (1942) in which a new hematocrit tube was described. This tube has proved so satisfactory, and in some respects superior to the hematocrit tubes in common use, that it is thought wise to draw it to the attention of workers in this country.

A diagram of this hematocrit tube is given in Fig. 1. The merits of this tube are that it requires such a small quantity of blood and that it has an enclosed end, and thus leakage during centrifugation, which so often occurs in the many modifications of the open end tubes, is completely prevented. The Wintrobe hematocrit tube also has a sealed end, but requires more blood than is often available from small animals or even large animals unless venepuncture is used. The size of the tube is such that it fits into a standard 50 c.c. centrifuge cup with a reducing cap. The capillary bore stem may be made in a variety of sizes depending on the amount of blood available. The author has two sizes for use in determining the packed cell volume of small laboratory animals. In one, the calibrated stem contains 20 c. mm., and the other 0.1 c.c. to be used with 20 c. mm. and 0.1 c.c. of blood respectively. The stem is divided into 50 equal parts, thus permitting the determination of the packed cell volume within 0.5 per cent by estimating the nearest quarter of a division. The volume of the packed cells read directly from the stem of the tube multiplied by two gives the volume of red cells in percentage. If desirable, the stem may be calibrated from 0 to 10 instead of 0 to 5 as here indicated, in which case the percentage of packed cell volume may be read directly.

The method of the author has been as follows: Deliver about 3 c.c. of isotonic saline into the hematocrit tube by means of a syringe with a 5 inch, 18 gauge needle which permits filling the tube from the bottom of the stem. If the hematocrit tube is a 0.1 c.c. one, deliver 0.1 c.c. of blood into the saline from a pipette calibrated to contain 0.1 c.c.; rinse the pipette two or three times with the saline and centrifuge to constant volume. Usually centrifuging at 2000 r.p.m. for one hour is sufficient. The tube may be conveniently cleaned by flushing out the stem and cup by means of the syringe and long needle.

For some time 0.85 per cent saline has been considered isotonic to mammalian blood, but in light of the recent work of Aldred (1940), this figure needs revision upwards. Aldred by use of the vapor pressure technique has determined the osmotic pressure of the blood of various animals and found that in the majority of mammals 0.93 per cent saline is isotonic to blood. Grüneberg found 0.93 per cent saline isotonic to mouse blood. Which one of the saline solutions selected is probably not as important as is a uniform use of one or the

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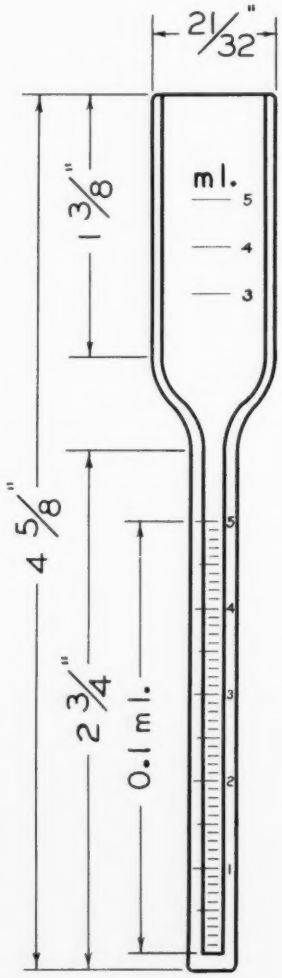
other, inasmuch as the centrifuge hematocrit is only a close approximation of the true red cell volume, and thus its use is for comparative purposes (Chapin and Ross, 1942).



HEMATOCRIT TUBE

Fig. 1.

Several sets of hematocrit tubes have been made for the author by Will Corporation, Rochester, N. Y., but any supply house should be able to furnish them to order. For precise work, the tubes may be checked for accuracy by calibrating the stem with mercury.

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RABBIT BOX*

HARRY J. WICK AND JOHN A. LEIGHTY, PH.D.,
INDIANAPOLIS, IND.

IN ORDER to facilitate the handling of large groups of rabbits in testing samples of human serum albumin for pyrogenic activity, a rabbit box has been

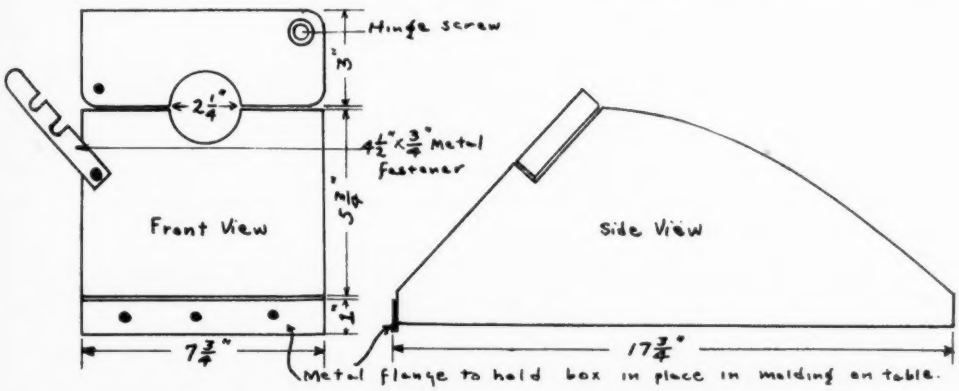

Fig. 1. Fig. 2.

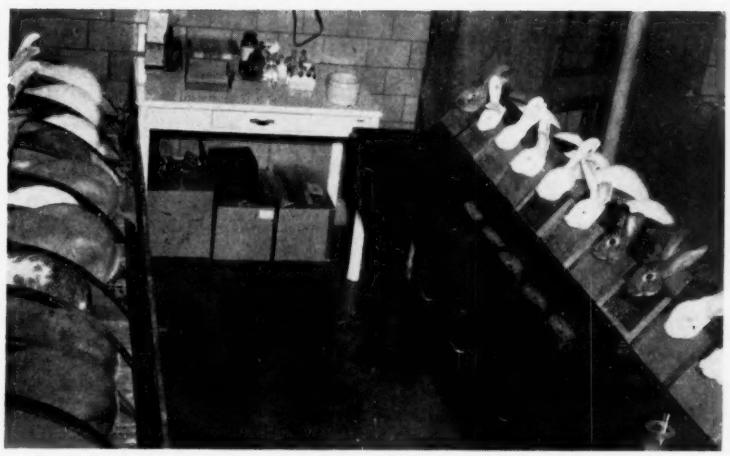

Fig. 3.

designed which offers some worth-while advantages. The animal remains in the box throughout the period of observation without being disturbed and without any noticeable discomfort. Incidentally, this type of box is also quite useful for skin-testing experiments, injection work, and blood-sampling experiments.

*From The Lilly Research Laboratories, Eli Lilly and Company.
The original idea for a box of this type was obtained while visiting the Massachusetts State Antitoxin and Vaccine Laboratory several months ago.
Received for publication, Sept. 25, 1943.

Fig. 1 shows a detailed drawing of the front of such a box, while Fig. 2 shows a side view. Seven-eighths inch wood is used for construction, and the rounded edges as shown are made to allow the boxes to be placed against one another in a row. The entire top of the box is open as can be seen in Fig. 3.

A SIMPLE RAPID METHOD FOR PARAFFIN TISSUE PREPARATIONS UTILIZING THE KAHN SHAKER*

LT. COLONEL FERDINAND C. HELWIG, M. C., A. U. S.

THE almost universal desire for rapid tissue diagnosis has resulted in numerous modifications of well-known methods of fixation, dehydration, and paraffin infiltration. In the hands of many workers these modifications, designed to decrease the time element, have frequently resulted in incomplete dehydration and infiltration, making cutting difficult. Moreover, the sections, thus obtained, all too often have shown too much shrinkage, poor staining qualities, and other objectionable features.

For some time now we have been employing a method in this laboratory which is not only rapid, but at the same time permits us to make histologic preparations that are easily sectioned and when stained show remarkably little shrinkage, less in fact than we have observed by the slow methods. Furthermore, we have eliminated certain common, more or less standard, steps which have likewise accelerated the preparation of very satisfactory sections.

It is possible by this method to make excellent sections, ready for examination within five to six hours after the tissue is received in the laboratory in the fresh state.

DESCRIPTION OF METHOD

Blocks not over one and one-half millimeters thick or one and one-half centimeters in diameter are cut from the tissue to be processed. If the tissue is fresh and unfixed, it can be suitably hardened by shaking it in the Kahn shaker in 20 per cent solution of formaldehyde for twenty or thirty minutes. We have been using small twenty cubic centimeter black screw-topped glass vials eight centimeters long and two centimeters in diameter. These vials are placed horizontally in the Kahn shaker so that the tissue will undergo constant agitation and will also be subjected to the largest, constantly changing, volume of fixing or dehydrating agent possible. If great haste is essential some time may be saved by immediate fixation in hot 4 per cent formaldehyde, but this method is undesirable since undue shrinkage and distortion result. Routine material can be fixed in the usual manner in 4 per cent formaldehyde solution for eighteen to twenty-four hours.

*From the Laboratory Service Army Air Forces Regional Station Hospital 1, Coral Gables, Fla.

Received for publication, Sept. 28, 1943.

After fixation the tissue blocks are blotted and the following solutions are employed:

SOLUTION	TIME
95 per cent Ethyl alcohol	30 minutes in Kahn shaker
Absolute alcohol	15 minutes in Kahn shaker
Acetone	15 minutes in Kahn shaker
Chloroform	30 minutes in Kahn shaker
Chloroform and soft paraffin (equal parts)	60 minutes in 57° C. incubator
Hard paraffin	60 minutes in 57° C. incubator

Absolute alcohol should be employed no more than three times and may then be used in place of 95 per cent alcohol for the first dehydration. Acetone should be used no more than three times. It may then be dehydrated over anhydrous copper sulfate and used over and over again.

The containers used for the fixing and dehydrating solution should contain at least fifteen cubic centimeters of the reagent for each tissue block. Tissues are embedded in hard paraffin nine parts and bayberry wax one part. A long block is made using a double "L" type lead embedding box. No fiber blocks are necessary since the paraffin bayberry wax mixture, when hardened into a block, can be screwed into the microtome and the blocks section easily. Routine sections are cut in our laboratory at six microns. Sections are then affixed to glass slides in the usual manner and stained by any of the methods suitable for paraffin preparations.

Comment.—The procedure just described is suitable for both surgical and necropsy material; it is saving of reagents and is rapid. In our hands this method has yielded uniformly good results over a long enough period of time and with a sufficiently wide variety of tissues to convince us that it is suitable for more widespread application.

A MEDIUM FOR DETERMINATION OF CITRATE UTILIZATION BY COLON BACTERIA*

CAPT. W. BLAKE CHRISTENSEN,† U. S. ARMY

DURING an investigation of certain unclassified dysentery bacilli in Tunisia, North Africa, it became desirable to test the reactions of the organisms on media containing citrate as a sole source of carbon. Since neither Simmons' citrate nor Koser's citrate nor the materials to make them up were available, it was necessary to devise a modification. A medium of the following composition was found suitable:

Ammonium sulfate (Merck Reagent)	1.0 Gm.
Magnesium sulfate (Merck U.S.P.)	0.2
Monopotassium *or* dipotassium phosphate (Merck Reagent)	1.0
Sodium citrate (Merck U.S.P.)	3.0
Sodium chloride (Merck Reagent)	5.0
Agar	20.0
Phenol red	0.012
Distilled water	1000 c.c.

The medium is heated to dissolve the agar, and the reaction is adjusted to pH 7. If monopotassium phosphate is used, alkali must be added, but if the dipotassium salt is used, acid must be added to bring the pH to 7. The medium is tubed and sterilized at 15 pounds pressure for twenty minutes and allowed to solidify in the form of slants. The fresh medium has a pale, yellow-orange color. If a liquid medium is desired, the agar may be omitted.

Organisms which utilize citrate as a sole source of carbon produce, within twenty-four hours on the solid medium, a yellow color in the butt and a very striking, intense violet color on the slant. Organisms which fail to utilize citrate leave the medium unchanged. In the liquid medium, citrate-positive organisms produce acid within twenty-four hours, with perhaps a narrow alkaline zone at the top of the tube. Within forty-eight hours, however, the entire body of the medium changes to the characteristic intense violet color. The presence of the indicator in the liquid medium eliminates the necessity of estimating growth by turbidity, as was done in the original Koser medium, and as large an inoculum as desired may be used.

Both liquid and solid media have been tested with 162 different strains of nonpathogenic and pathogenic colon organisms. The results are shown in Table I. It is seen that only organisms of the Aerobacter and colon intermediate groups, and *Salmonella typhi murium* utilized the citrate. Organisms of the Escherichia, paracolon Escherichia, and Shigella groups, and *Eberthella typhosa* failed to utilize the citrate. In the anaerogenic paracolon group only citrate-negative organisms were found, no doubt because of the small number of strains tested.

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†Chief of the Bacteriology Section, Sanitary Corps.

Though it would have been desirable to check organisms of the colon intermediate and the anaerogenic paracolon groups with standard citrate media to determine the possibility of false reactions, it was manifestly impossible to do so. However, the uniform negative results obtained with organisms known to be citrate-negative and the positive results with organisms known to be citrate-positive are ample evidence of the reliability of the medium.

TABLE I

CITRATE UTILIZATION BY COLON ORGANISMS ON THE MODIFIED CITRATE MEDIUM

ORGANISM	NO. OF STRAINS TESTED	CITRATE UTILIZATION	INDOLE	VOGES-PROSKAUER
Escherichia	46	−	+	−
Paracolon Escherichia	5	−	+	−
Colon	8	+	+	−
Intermediate	4	+	−	−
Aerobacter	24	+	−	+
Anaerogenic paracolon	2	−	+	−
	3	−	−	−
Various Shigella	23	−	+	−
Species	45	−	−	−
Salmonella typhi murium	1	+	−	−
Eberthella typhosa	1	−	−	−

− Negative.
+ Positive.

Considerable stress has been placed on the utilization of chemicals of the highest possible purity in a citrate medium. For this reason, some objection may be made to the use of the two U.S.P. salts in the medium. However, even if the impurities consisted entirely of a compound utilizable by citrate-negative organisms, and if the utilization brought about the same type of reaction as true citrate fermentation (assumptions which are in themselves very unlikely), there still would not be enough of the contaminating compound present to produce enough alkali to change the color of the medium. Impurities would hardly be present in a concentration to exceed 0.0005 M. (based on stated impurities in the Merck Index, 1941). Such a concentration is well below the known affinity constants of enzymes, and the compound would not be used at all, or would be used so slowly that appreciable growth would not take place.

The medium described consists of materials that are cheap and readily available in most laboratories. While it will probably not replace standard media on the market, it will fill the needs of laboratories which use such a medium rarely and thus have no reason to lay in a stock of the prepared product.

A MULTIPLE RABBIT HOLDER*

EDWIN P. LAUG, PH.D., WASHINGTON, D. C.

IN STUDIES requiring the immobilization of rabbits for extended periods, it has often been found that the usual methods are undesirable, because they may produce either (1) stasis from the tie strings (even when padded); or (2) broken legs and backs due to struggling.

These difficulties have been overcome through the design of a holder which immobilizes the animal after the manner of a head and hip stock, but without restriction to the movement of fore or hind limbs. The animal rests comfortably on its belly; but because of the fact that its hind quarters are held rigidly, no leeway is permitted for sufficient arching of the back or other motion to cause injuries which so frequently result. The type of holder described here has been in use for nearly two years; during this time approximately 2,500 rabbits have been kept in the holders for periods varying from one to twenty-four hours. In this extensive experience, practically no injuries have resulted when the animals were properly placed in the stocks; occasionally accidents have occurred where the confining jaws were improperly adjusted so that the animal managed to free either the head or the hips. Constructional details: In Fig. 1 are reproduced the constructional details for a 6-rabbit holder. For long service the use of a hardwood such as birch or maple is recommended, particularly in those parts subject to frictional wear. It is not desirable to describe the construction in any detail, since it is believed that the pictures and drawing are self-explanatory. Several features, however, may well be stressed:

The head piece is stationary, being part of the framework of the holder. Both jaws of the head piece are removable, and this facilitates cleaning. In practice it has been found most essential that the lowest point in the opening of the lower jaw piece be fixed not higher than one inch above the table level upon which the animal rests; otherwise the head is elevated too much and choking results. Adjustments for size of the head opening are made by locking the upper jaw piece in proper position through a series of nail holes, one of which passes through the stock frame. The hip stock is adjustable for the size of the animal's hips in a manner similar to the head stock. In addition, however, the entire hip stock is movable forward and backward, sliding in two groove tracks, one on each side. Thus adjustments for the length of the animal can be made quickly. As in the case of the lower jaw piece of the head stock, it is very essential that the lowest point of the lower hip jaw be not further than ½ to ¾ inches above table level. Failure to observe this dimension leads to serious constriction, and bladder injury often results. The openings of the head and hip jaws have been adjusted to accommodate animals weighing 2 kilograms or over.

*From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency.

Received for publication, Oct. 9, 1943.

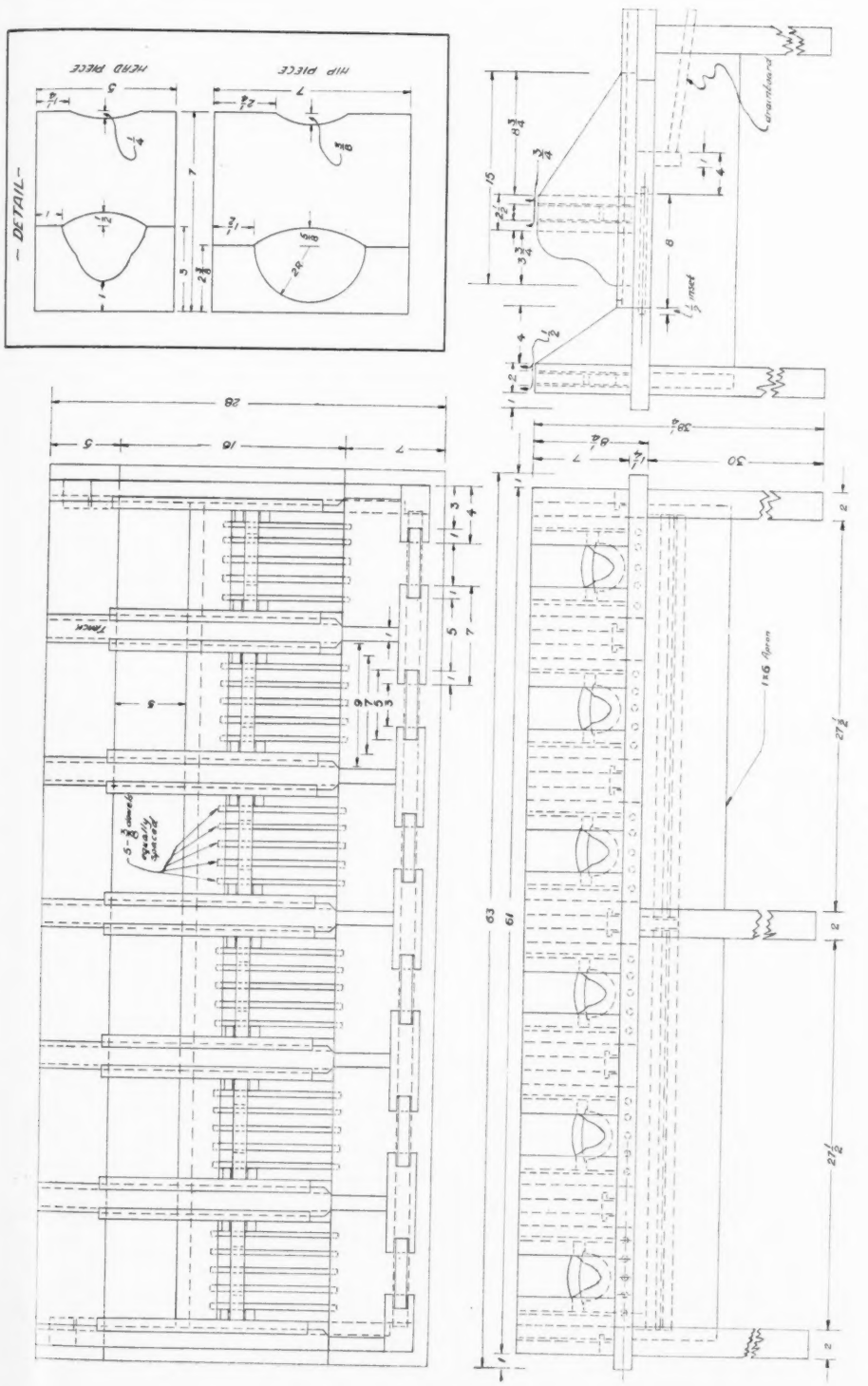

Fig. 1.

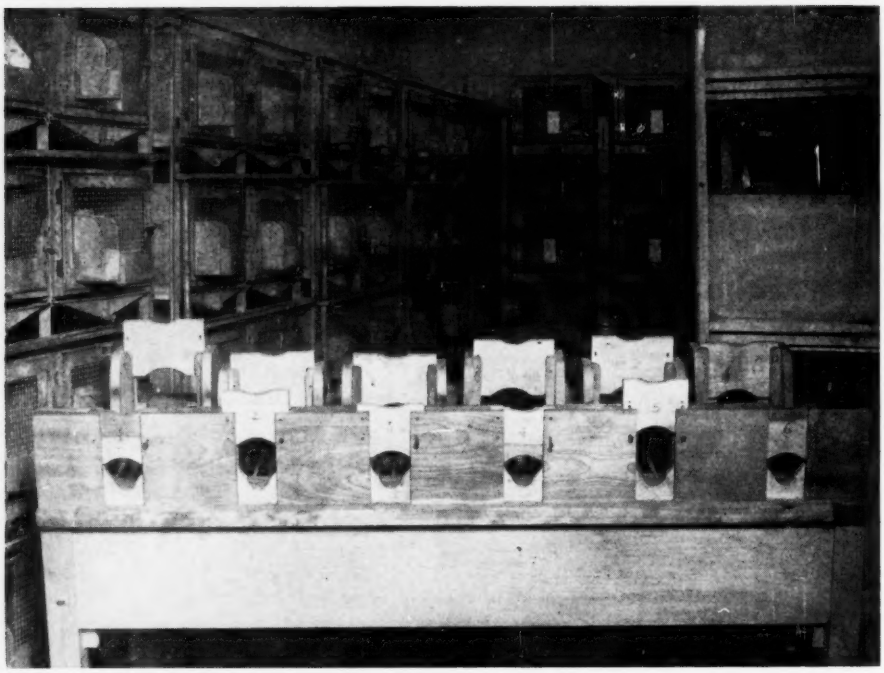

Fig. 2.

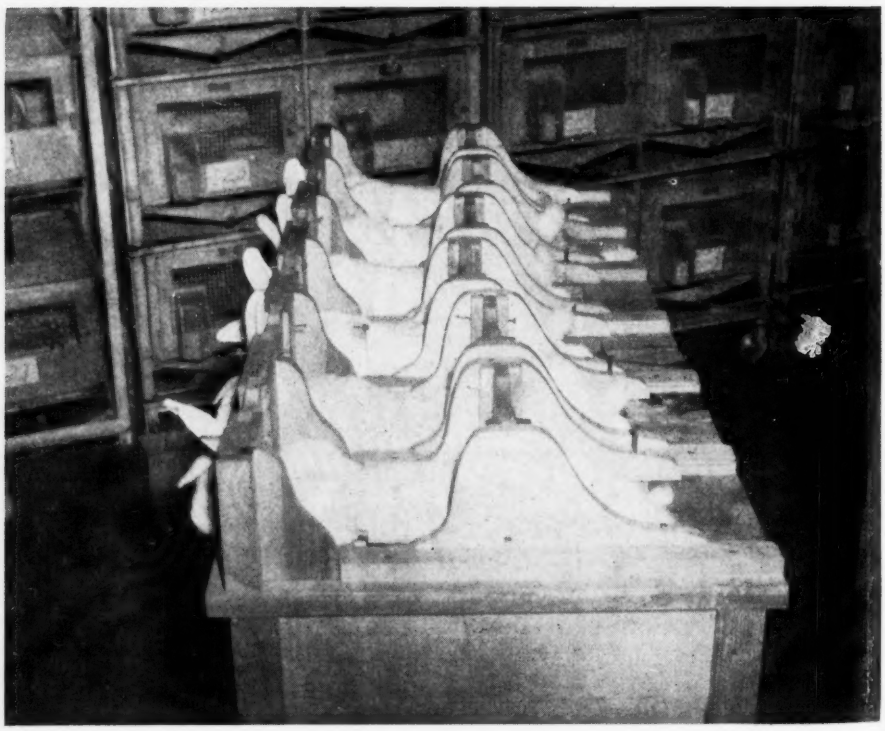

Fig. 3.

The shape of the fixed lower jaws of both head and hip stocks will accommodate animals weighing over 5 kilograms. For small animals, the top jaws of both head and hip stocks have been made reversible (see Fig. 1). For very small rabbits (less than 2 kilograms) further modification of the shape of the top jaws would be necessary.

The belly of the rabbit rests on a framework of 5 dowel pins. This feature is extremely useful where fluids are applied to the skin of the back. Sometimes these tend to run off and without the open construction would collect under the belly of the animal.

At the back of the holder, provision for removal of excreta has been made by installing a removable drain board as shown. Quantitative collection of urine and feces can, of course, also be made by substituting for the drain board a sliding rack for glass baking dishes and a baffle for deflecting urine.

The length of time that an animal can be kept immobilized becomes primarily a problem of a need for food and water. Usually rabbits will not eat voluntarily when thus confined. While held in the holder, however, it is relatively easy to pass a stomach tube.

While the author has had no experience with other animals, it would seem feasible to modify the sizes of the head and hip jaws to accommodate dogs and cats.

A RAPID METHOD FOR ESTIMATING THE BACTERIOSTATIC ACTIVITY OF DISINFECTANTS*

W. A. RANDALL, PH.D., AND C. W. PRICE, A.B., WASHINGTON, D. C.

THE ability of certain bacteria to reduce nitrate to nitrite rapidly has been known for some time. Stickland,[1] in 1930, demonstrated this phenomenon in *Escherichia coli* cultures, and Randall and Reedy[2] later found that many bacterial species possessed this property. Inasmuch as reduction occurs only during the active growth phases, it may be assumed that immediate injury to the cell would result in changes which might be reflected in the reducing ability of the culture. With this in mind, investigations designed to illustrate effects of bacteriostatic agents upon the cell's ability to reduce nitrates were conducted.

The procedure consisted merely of allowing various dilutions of the antiseptic being tested to remain in contact with a broth culture of the test organism for a period of time, adding KNO_3, and, after an incubation period, testing for the presence of nitrite. The organism used was *Staphylococcus aureus,* FDA strain 209.

A twenty-four hour broth culture of the test organism is added in 0.5 c.c. portions to 0.5 c.c. of serial twofold dilutions of the antiseptic in small test tubes and permitted to remain in contact at room temperature for five minutes. Two-tenths milliliter of 1 per cent aqueous solution of potassium nitrate is added; the tubes are thoroughly shaken and incubated at 37° C. for thirty minutes. To each tube 0.2 c.c. 6/N HCl, 0.2 c.c. of 0.1 per cent N(1-naphthyl) ethylene diamine dihydrochloride, and 1.0 c.c. of 0.2 per cent p-amino benzene sulfonamide (sulfanilamide) are added, and five minutes are allowed for the red coloration to develop, which action occurs in the presence of nitrites. The highest dilution in which nitrite production has been completely inhibited is recorded as the end point.

Bacteriostatic activity is determined at the same time by the use of 0.5 c.c. of a similar twofold dilution of the antiseptic in FDA broth and by the addition of 0.5 c.c. of a one to one-million dilution of the test organism as inoculum. The tubes are incubated at 37° C. for forty-eight hours, and the highest dilution showing complete inhibition is recorded as the bacteriostatic end point. All of the broth in tubes showing complete inhibition is then subcultured to determine the lethal concentration.

Many commercial compounds were examined by the foregoing methods, and the correlation was found to be very close, considering the error inherent in the twofold dilution system. A nitrate-nitrite reduction bacteriostatic ratio apparently exists and can be determined for each type of compound. By applying this factor, one can determine with reasonable accuracy the bacteriostatic end point.

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Table I reports the results from the examination of twenty-seven disinfectants to illustrate the correlation between the nitrate reduction factor and the bacteriostatic and lethal end points. The disinfectants reported as Class "A" are the quaternary ammonium compounds of the three main types, and the class "B" are the newer phenolic type disinfectants.

TABLE I

TYPE	SAMPLE NO.	NITRATE TITER	INHIB.	LETHAL	FACTOR	
					NO. 3 INHIB.	NO. 3 LETHAL
CLASS "A"						
1	1- 5	4000	32000	16000	8	4
2	6-11	4000	32000	32000	8	8
3	12-14	2000	16000	16000	8	8
CLASS "B"						
1	1- 7	4000	8000	8000	2	2
2	8- 9	2000	8000	8000	4	4
	10	1000	4000	4000	4	4
3	11-13	2000	8000	8000	4	4

Inspection of Table I reveals that in any given class and type of compound a nitrate-reducing bacteriostatic or nitrate-reducing lethal factor which remains constant for that particular type of disinfectant can be determined. Sample No. 10, of Type 2, Class "B," represents a disinfectant which failed to meet the Federal specifications under which it was purchased. The lack of disinfectant power is reflected in the inhibition of nitrate reduction, however, to the same extent as in the bacteriostatic activity, and the factor remains the same for the members of this particular group which were of standard strength.

In the course of the investigation, many other disinfectants were examined, and their ability to inhibit nitrate reduction was found to correlate with their bacteriostatic powers. An antiseptic containing nitrites would of course invalidate the test for that particular compound, but as yet none has been encountered, nor have any disinfectants contained ingredients which gave color reactions with the reagents employed.

Three practical uses of the test, as described, suggest themselves:

1. In bacteriostatic studies on similar chemical compounds, as a rapid method of eliminating weakly bacteriostatic substances.

2. In sterility control as a preliminary test for bacteriostatic activities of the product being examined.

3. As a possible control test during the manufacture of disinfectants and antiseptics.

Summary.—A rapid method is described for the estimation of the general bacteriostatic and bactericidal efficiency of antiseptics, in which the ability of the antiseptic to inhibit nitrate-nitrite reduction is determined.

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A SOLID MEDIUM FOR THE TRANSPORTATION OF DELAYED GONOCOCCUS CULTURES*

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THE provision of reasonably adequate facilities for gonococcus cultures in a state laboratory requires the solution of several problems: First of all, the establishment of reliable local laboratory service and, second, the development of a method for sending gonococcus cultures through the mail. The former depends on a simple method for teaching technicians, many of whom are not familiar with bacteriologic procedures, to do such work. The latter depends on the establishment of a method for keeping the gonococci alive and holding back contaminating organisms. Many such methods have been described, using special liquid media, ice or dry ice refrigeration, or "thermos" bottle containers.[3, 4, 5, 8, 9, 11, 12, 13, 19, 21, 24, 26]

It is well known that diagnosis of gonorrhea in the male can be made by examination of suitable smears. In the female, however, especially in chronic or treated cases, a few doses of sulfonamide may render the smears negative, while many viable organisms are present, or the large number of contaminating bacteria may interfere with the interpretation of a smear, even if care is taken to take these smears from the cervical canal.

The value of gonococcus culture is well known. Thomas[22-24] has summarized this work through 1941. Even with the relatively ineffective means now available, cultures yield two to three times as many positive diagnoses as smears in chronic or treated female cases.[14, 15, 16, 17, 20, 25]

North Carolina is a rural state, approximately 550 miles wide, with considerable variations in temperature, ranging from zero in the mountains in winter to 103° F. during the summer. While 87 of its 100 counties have organized full-time health departments, most of the culture material will come from well-trained physicians in urban centers, country doctors and nurses in rural centers, and will take from two to three days to reach the laboratory.

For this reason, it seemed inadvisable to use liquid medium or to refrigerate specimens with ice or dry ice. We have, therefore, attempted to develop a suitable solid medium in tubes to be inoculated with material collected on full size applicator swabs. This paper is a preliminary report of the results obtained using such a medium.

Preliminary work was done on 25 specimens, secured from untreated prostitutes from the Woman's Prison in Raleigh and from nearby clinics sufficiently close to Raleigh that the specimens might be brought by messenger. Later, 117 specimens were secured from 85 patients in a well-directed Gonorrheal Clinic, conducted by Dr. Margery J. Lord, City Health Officer, Asheville, N. C., some 270 miles from Raleigh. It was felt that cultures which could survive

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this long trek across the country in the mails should be a fair test of a method suitable for the entire state.

The mailing media consisted of numerous combinations of blood with and without the addition of dyes and other substances to inhibit contaminants.[3, 4, 5, 6, 10, 21] Each one was tested for mailing qualities, inhibition of contamination, and preservation of the gonococcus. Those combinations containing less than 1 per cent agar did not stand transportation and were eliminated. In the development of the media various concentrations of gelatin and agar were tried, the former ranging from 2 per cent to 24 per cent, the latter from 0.1 per cent to 2.5 per cent. Agar by itself is too brittle; when a swab is introduced into it, even if the medium contains up to 50 per cent blood, the agar is broken up with large air spaces resulting. It was found that the addition of 4 per cent gelatin to 2 per cent agar produced a medium of smooth composition, regardless of the quantity of blood, into which a swab can be introduced and withdrawn with ease.

Each mailing medium was tested with a known strain or several known strains of the gonococcus. The media containing inhibiting agents were tested with known cultures of the colon bacillus, *Staphylococcus aureus*, and proteus. Concentrations of dye which do not kill the gonococcus are for the most part ineffective against the latter organisms. Almost all the combinations showed some inhibition of contaminants but did not exhibit the marked inhibition which is probably desirable. A few tests were made with tyrothricin[2, 21] which seemed no better or no worse than Nile blue A.[10] Crystal violet of suitable dye content as suggested by Cox[4-6] was tested also, and a few tests using brilliant green, alone or in combination with the other dyes, were tried. (Dr. Cox very kindly sent us some of his media to try.) It seems that the degree of contamination in the final cultures depends more upon the bacterial flora of the patient than upon the inhibiting agent used. The isolation of the gonococcus was made both from heavily contaminated and slightly contaminated plates. We do not know how much the contaminants interfere with the preservation of the gonococcus. It does not seem that sulfonamide treatment of the patient interferred to any large degree with the isolation of the organisms.[19] Para-amino benzoic acid was introduced into one set of mailing media, but better results were obtained without this addition. The medium which we have finally determined as the most suitable to date consists of the following: proteose-agar No. 3 (Difco), 4.5 per cent plus additional shredded plain agar to make a final concentration of 2 per cent, 4 per cent gelatin, 0.24 per cent Nile blue A, and 20 per cent chocolate blood. The medium is very smooth, easy to penetrate with a swab, and supports the growth of the gonococcus luxuriantly. It seems to be more satisfactory with Nile blue A than with gentian violet, tyrothricin or brilliant green, although it must be stressed again that all evidence in our series of cultures point to the fact that no satisfactory bacteriostatic agent has yet been tested, the degree of contamination depending for the most part upon the patient's vaginal flora.

Cultures and smears were taken as the patients were examined in the routine Friday night Asheville Gonorrheal Clinic. (We are grateful to Dr. Lord for this clinical material.) Some of the patients were new acute cases, some were under treatment, others were recurring cases which had received

treatment sometime before, and a few were new contacts. Swabs were taken from the urethra, using pressure on the Skene's Glands to milk out any material present. Smears and cultures were made from these swabs. A speculum was then inserted in the vagina, the cervix cleaned off with a cotton swab, the mucous plug in the cervix expressed, and another smear and culture were made with a swab from the cervical canal. The clinical condition of the patient was carefully noted.[15] This procedure is followed routinely in the clinic and gives approximately three times as many positive cultures as smears.[16] To test the mailing media, additional swabs were taken from the cervix and in some instances also from the urethra, using sterile forceps to break off the swabs and push them down into the mailing tubes.

After the clinic, that is about ten o'clock at night, these mailing tubes were labeled and packaged to be sent through the mail the next morning to the State Laboratory of Hygiene in Raleigh. Occasionally the package was received at the laboratory on Sunday, in which case cultures were made immediately. Generally, however, the tubes were received following the sorting of the mail on Monday and were cultured Monday afternoon, approximately three days after the swabs had been taken from the patients. In one series five days elapsed. In the group of cultures tested before this routine was established in the Asheville Clinic, specimens remained in the mailing media from twenty-four hours to six days. The mail between two points probably never takes that long, but cultures were purposely held in the laboratory to see how long they would remain positive.

The swabs were removed with sterile forceps, streaked onto Peizer's medium[18] and onto chocolate agar[2] plates. These plates were sealed in a jar containing a moist piece of cotton and a lighted candle and incubated for two days at 36° C. Some of the swabs were suspended in ½ c.c. of 2 per cent proteose peptone No. 3 solution, well-shaken, and cultures were made from this suspension. It was noted that growth on the surface of some of the mailing media looked suspicious for the gonococcus, and so these tubes were incubated in the jars with the subcultures after the swabs had been removed and were tested by the oxidase reaction two days later. A few positives were obtained by this method.

After forty-eight hours, the plates were examined by means of the oxidase reaction and Gram-stained smears. Doubtful strains were tested by fermentation reactions. The plates were read carefully for the degree of contamination as well as for the number of positive colonies which in most cases was low, occasionally one or two oxidase positive colonies only appearing on the surface of the plates. The results obtained in this laboratory were checked against those control cultures and smears which had been made in the Asheville City Health Department Laboratory at the same time.

Table I shows the results from our series of 142 cases. Of the 25 specimens received by messenger from patients presumably with gonorrhea, 10 showed immediate positive control cultures, while 8 delayed cultures were positive, 3 after one, 3 after two, 1 after three, and 1 after four days. Most of them remained on the desk in the laboratory for the specified period of time, while a few were mailed in from near-by towns.

Forty-four specimens from cases of acute gonorrhea diagnosed by positive smear and culture were mailed to the laboratory. The immediate control cultures of the 44 cases were positive, and 31 of the delayed cultures were positive, 2 after two, 20 after three, 6 after four, and 3 after five days in the mail.

Sixty-nine mailed specimens were received from cases of chronic or treated gonorrhea. None of these showed positive immediate control cultures, 12 of them showed positive cultures from the specimens which were mailed to the laboratory, 2 after two and 10 after three days. These 12 positives possibly may be accounted for by the fact that the mailing medium may absorb some of the drugs used in treatment which may be affecting the direct growth of the gonococcus, or by the fact that in these cases there are present only a few gonococci which may be picked up on one swab and not on another or may even have been missed on the direct cultures. Four cultures from clinically negative contacts were negative throughout. These results indicate that the mailing medium is quite satisfactory for isolating the gonococcus after several days have elapsed. Since the greatest number of cultures reached the laboratory in three days, this accounts for the seemingly large number of positives obtained in that length of time. In the series of 117 mailed cultures, a total of 16 were received in two days with 3 positive isolations, 73 were received in three days with 31 positive isolations, 13 were received in four days with 7 positive isolations, and 15 were received in five days with 3 positive isolations.

TABLE I

NUMBER OF CASES	DIAGNOSIS	IMMEDIATE CULTURES		DELAYED CULTURES						
						DAY OF CULTURE				
		NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	1	2	3	4	5
25 Messenger Specimens	Presumably gonorrhea	15	10	17	8	3	3	1	1	0
44 Mailed Specimens	Acute gonorrhea	0	44	13	31	0	2	20	6	3
69 Mailed Specimens	Chronic or treated gonorrhea	69	0	57	12	0	2	10	0	0
4 Mailed Specimens	Contacts-Clinically Negative	4	0	4	0					
142 Total		88	54	91	51	3	7	31	7	3

SUMMARY

A solid medium suitable for the transportation by mail of specimens for gonococcus culture has been described with the preliminary report on the use of such a medium. This solid medium stands transportation, is simple to use, and gives a fair percentage of isolations of the gonococcus.

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CONVENIENT ASSAY METHOD FOR PENICILLIN AND SIMILAR SUBSTANCES*

Use of Large Plates and Filter Paper Circles

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THE agar cup method for evaluating the antibacterial potency of solutions is favored by many because of its simplicity and reliability. The procedure described here retains the advantages of this standard method.

The details of determining unitage of penicillin by the agar cup method (using Petri dishes) have been adequately described by Abraham et al. (1941), Foster (1942), and Foster and Woodruff (1943). The estimation of potency in penicillin solutions by the Oxford cup plate method, as carried out at the present time, involves the use of many Petri dishes in order to observe the extent of zones of inhibition over a suitable range of dilutions.

In an attempt to diminish the time and labor involved both in conducting the test and in the preparation of glassware, we have adopted the use of flat Pyrex baking dishes as culture dishes. Dishes of two sizes have been used, 6½ inches by 10½ inches by 2 inches and 12½ inches by 8 inches by 2 inches, the latter size proving the more convenient. These are fitted with plate glass covers cut to the proper size and fastened to the plate flanges with cellulose tape before incubation is begun.

PROCEDURE

The smaller plates are prepared by pouring into them 100 c.c. of melted cooled agar seeded with 0.3 c.c. of an 18-hour culture of the Oxford test strain (*Staphylococcus aureus* strain H).

The larger culture dishes are made with 147 c.c. of agar and a 0.45 c.c. of the above inoculum. Satisfactory results have also been obtained using agar seeded with *Bacillus subtilis* spores according to the method of Foster (1942). Evaporation of excess moisture from the surface of the agar before use can be accomplished by inverting the plates and propping them open with a cork placed between one edge and the lid.

Tests are carried out by a wet filter paper method similar to that of Lamanna and Shapiro (1943). Sterile 1 cm. circles of an absorbent filter paper (Fisher No. 9-795) are dipped with flamed forceps into tubes containing the dilutions of penicillin to be tested, drained momentarily against the side of the tube and placed in rows at suitable intervals on the agar surface. A similar method has recently been used by Dowdy et al. whose work was cited by Foster and Woodruff (1943).

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A standard of known unitage is run on each plate as a control. We have found that the zone of inhibition produced by a 1 cm. filter paper circle impregnated with fluid containing 1 Florey unit per c.c. is somewhat less in diameter than that resulting when the Oxford cup plate method is used with the same standard, being, in our case, about 18 to 20 mm. The diameter of the filter paper is included in measuring the zone of inhibition.

A number of advantages are inherent in the use of the present method other than the ease of preparation of sterile apparatus. Testing numerous samples on the same culture surface increases the over-all uniformity of the assay method. Experience has also shown that the zones can be measured more quickly, there being more on each plate. A larger number of samples can be assayed by this method than by other methods hitherto described, there being comparatively little time consumed other than in making dilutions.

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PERMANENT METACHROMATIC STAINING OF GASTRIC MUCUS SMEARS*

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THE staining of different tissue components in different colors by a single dye is called metachromasia. Thus, if a dilute solution of a basic dye like toluidine blue is used to stain a tissue section, some of the components will be stained blue, whereas others, called chromotropic, will be stained purplish red. Typical examples of metachromasia are afforded by mucin, cartilage, amyloid, and the material constituting mast cell granules. The obvious advantage of metachromatic staining lies in the fact that it furnishes a degree of differentiation by means of one dye alone; the outstanding difficulty encountered with this technique is its lack of permanence following dehydration, a difficulty which is recognized in all textbooks of histologic technique. In our experience with the usual technique, applied to smears of mucous secretion, even air drying resulted in immediate conversion of the metachromatic purple to an undifferentiated blue. After numerous trials we finally evolved a technique which overcomes this difficulty, and we are reporting our method herewith.

The principles underlying metachromasia have been investigated mainly by Lison,[4] who found that sulfuric acid esters of high molecular weight are the most potent chromotropic materials. For instance, heparin appears to be responsible for the metachromatic staining of the mast cell granules (Holmgren and Wilander[2]); mucoitin and chondroitin sulfuric acids are the groups responsible for the chromotropic properties of mucoproteins found in mucus and cartilage. The protein moiety of the mucoproteins appears to exert no influence on the chromotropic character of these substances. The reaction is so specific that, according to Lison, it can be used as a histochemical test for the detection of the presence of these characteristic groups. Efforts to understand the nature of this color change have not been successful, however. Lison believed that there exists in the dye solution an equilibrium between the metachromatic and the normal forms, but he did not try to define the chemical basis for this differentiation; contact with a chromotropic substance can shift this equilibrium towards the metachromatic side. Other explanations have been based on differences in dye concentration between the two forms (Bank and Bungenberg de Jong[1]) and intramolecular rearrangements (Kelly and Miller[3]). None of these investigators, however, was able to explain the disappearance of metachromasia which results from treatment with alcohol or from drying.

It was our purpose to find a method for staining smears of gastric mucus so that the cellular structures would be visible by contrast against the back

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ground of mucous material. Mucicarmine alone affords no such differentiation, and when used after nuclear staining with hematoxylin, the counterstaining is erratic and generally unsatisfactory. Metachromatic staining with the usual toluidine blue technique yielded satisfactory contrasts in wet preparations, but, in keeping with general experience, the metachromatic color disappeared as soon as the smear was dried or treated in the usual way for mounting in Canada balsam; since we were no more fortunate than other investigators in formulating an hypothesis to explain this loss of metachromasia, we approached the problem in a purely empirical way and finally succeeded in evolving a technique for the preparation of permanent metachromatically stained smears of gastric mucus. Specimens thus stained ten months ago still retain their characteristic metachromatic stain.

Our procedure is as follows: Smears of fresh mucus, collected from gastric pouch dogs, are left to dry at room temperature and then fixed by flaming. The slide is then placed in the staining solution, where it is agitated vigorously for fifteen seconds and then left undisturbed for one minute more. The staining solution is prepared as follows: 1 Gm. of toluidine blue (National Aniline, certified) is dissolved in 90 c.c. of distilled water and filtered; to it are added 0.50 c.c. of HCl (specific gravity 1.18), 0.90 c.c. of H_2SO_4 (specific gravity 1.84), and 10 c.c. of a 1 per cent solution of alum; finally 5 c.c. of 95 per cent alcohol are added to the entire mixture. From the staining solution the slide is taken through two changes of 1 per cent HCl (1 c.c. of HCl, specific gravity 1.18, to 9 c.c. of distilled water), being washed for about ten seconds in each change so that no more dye comes off the slide. The slide is then immersed for one minute in a 5 per cent aqueous solution of $HgCl_2$, washed vigorously in two changes of 95 per cent alcohol (five seconds each), and cleared in two changes of xylol. The slide should be agitated vigorously in the first change of xylol to eliminate the alcohol. Complete removal of the alcohol has occurred when the temporary blue discoloration caused by the alcohol treatment is replaced by a purple color. The slide is left for at least two minutes in the second change of xylol. Finally, the smear is mounted in Canada balsam and a cover slip is applied.

The mucus is spread on the slide gently with the aid of a glass rod, thus avoiding disruption of groups of cells and expression of intracellular mucus. The use of chemical fixatives at this stage was discarded after repeated trials with various agents. Formalin and Susa fixative both prevented metachromatic staining, saturated $HgCl_2$ removed material from the slide and impeded the fixation of the dye after staining, acetic acid distorted the mucus by irregular shrinkage, and alcohol caused deformation of the nuclear material.

Mild acidification of the dye solution with hydrochloric acid yielded a marked increase in color contrast between the mucus and the nuclear material, but excessive acidification led to shrinkage and destruction of the nuclei. Corresponding intensification of contrast was not obtained with other acids; acetic acid, on the contrary, actually prevented the color differentiation. The addition of sulfuric acid to the dye solution, however, appeared to increase considerably the intensity and substantiality of the staining. In this connection it was observed that the use of hydrochloric acid alone resulted in an appreciable loss of dye during the later steps of the method, whereas the combination of hydro-

chloric and sulfuric acids prevented such loss. Addition of alum prevented loss of mucus from the slide and brought out sharply the intracellular mucus in a striking color contrast. The use of mildly acidulated water for washing enhanced the staining of the nuclear and cytoplasmic substances, whereas pure water imparted a purplish color to these materials, and more highly acid solutions led to a marked loss of dye.

None of the foregoing procedures, however, yielded any degree of permanence of metachromasia, unless the smear was fixed *after* staining. To this end we tried formalin (10 per cent), Susa, formol-alcohol, and saturated solution of $HgCl_2$; only the last one of these gave the desired result. Its use *before* staining, however, was ineffective. The metachromasia could thus be preserved throughout the usual treatment with alcohol and xylol, and subsequent mounting in balsam even improved the brilliancy of the colors.

The finished preparations show both extra and intracellular mucus stained red to purplish red. The cells, on the other hand, are invariably stained blue, the nuclei being clearly differentiated from the cytoplasm by the greater depth of blue color. Contrast between intracellular mucus and cytoplasm is very striking. Smears prepared according to this technique permit a study of fine cellular details with the oil immersion lens.

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CHEMICAL

THE ASCORBIC ACID SATURATION TEST
(A Preliminary Report)

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SINCE the isolation of vitamin C in 1928 by Szent-Györgyi,[1] many investigators have been interested in the chemical determination of this vitamin in blood and other body fluids. Various authors have shown that the normal adult should have a daily intake of at least 50 mg.[2-5] Levcowich and Batchelder[3] believe that the calculated ascorbic acid content of foods in a freely chosen diet may be overestimated and give a false sense of security as adequateness of vitamin C. According to Heinemann,[2] at least 0.8 mg. of ascorbic acid per kilogram body weight is used by a normal vitamin C-saturated individual; however, 0.4 mg. or less daily is probably sufficient to prevent scurvy. To maintain tissue saturation, 1.7 to 2.0 mg. per kilogram body weight is required.[5]

An individual's past intake of vitamin C may be decided either with an estimation of the ascorbic acid content of the blood or with the use of a saturation test, in which a known quantity of vitamin C is administered and the rate of excretion in the urine for a standard period of time is determined. The saturation test is based on the assumption that a past deficiency of vitamin C results in a low concentration in the tissues. Therefore, when this vitamin is administered (orally or intravenously), the tissues are capable of rapidly removing it from the circulating blood and a very small quantity is excreted in the urine. On the other hand, if the tissues are saturated, then, when the test dose is administered, the ascorbic acid will be removed from the blood and excreted in the urine in varying quantities, depending on the degree of tissue saturation. Lewis et al.[6] found that the renal threshold for ascorbic acid in twelve normal adults ranged from 1.1 to 1.8 mg. per 100 c.c. Faulkner and Taylor[7] agree that this threshold is in the vicinity of 1.40 mg. per 100 c.c. of serum.

A saturation test may be preferable to a blood determination because a venipuncture is not required. Furthermore, as stated by Harris,[11] "a subnormal level in the blood probably gives less clear quantitative evidence of the exact extent of the deficit. . . ."

There are objections to the use of the term "tissue saturation" as applied to ascorbic acid concentration in tissues. Hou[8] observed that ascorbutic tissues were capable of removing more ascorbic acid from a solution than normal tissues.

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The saturation test described by Harris[9] consists of a test dose of 700 mg. per 10 stone body weight (140 pounds), which is administered orally at 10:30 A.M. During the peak of the vitamin excretion, 2 to 4:30 P.M., a 2½-hour urine specimen is collected. This test dose is repeated daily until the body's reserves approach saturation, which is indicated by the rise of the ascorbic acid excretion. The saturated individual should excrete at least 50 mg. per 10 stone weight for the 2½-hour period on the second day, while an inadequate vitamin C intake is manifested by requiring a longer period of time before obtaining a satisfactory response to the test dose.

Other workers have recommended a 24-hour saturation test. Also, for this purpose, ascorbic acid may be used intravenously. According to Hawley and Stephens,[10] in saturated subjects the maximum excretion, after oral administration, is obtained during the third, fourth, fifth, and sixth hours; while after intravenous use it occurs during the first and second hours.

METHOD OF STUDY

In this study the subejcts were requested to avoid eating fruit, fruit juices, and those foods high in vitamin C, immediately before and during the test. After breakfast, the bladder was emptied, and the subject was given 200 mg. ascorbic acid, and a glass of water. (The tablets were swallowed in the presence of the author.) The bladder was emptied every hour for six hours. The majority of the urine specimens were titrated* (with a standardized, purified sodium 2,6 dichlorophenol indophenol) within fifteen to twenty minutes after voiding. In one part of this study the specimens were collected over a 24-hour period. The night specimens were preserved with acetic acid and titrated the following morning.

All were adults; the majority were between 20 to 30 years of age.

One group of seven women and eight men, presenting a history of having had considerable fresh fruits and vegetables for at least one month prior to the test, were selected for the first part of this study. These were considered to be average normal adults on an adequate vitamin C diet. A second group of eighteen men, presenting the history of having had a limited quantity of fruit and fresh vegetables, were selected on the assumption that they had an inadequate vitamin C diet. The third part of the investigation consisted of four patients suspected of a definite vitamin C deficiency. An additional study was undertaken on three men to determine the excretion of ascorbic acid during a twenty-four-hour period, without and with the test dose.

RESULTS

Fifteen adults, considered to have had an adequate vitamin C diet, received an oral administration of 200 mg. of ascorbic acid (Table I). The average total excretion for the four-hour period was 27 mg.; the total for the six-hour period was 50 mg. The range for the four-hour test was 17.3 to 71.6 mg., while the six-hour range was from 27.9 to 82.3 mg.

*Ascorbic acid, which is a strong reducing agent, is capable of decolorizing sodium 2,6 dichlorophenol indophenol. It is well understood that other substances may also be present in urine possessing this property. These substances can be removed with mercuric or barium acetate. For clinical purposes, this does not appear to be necessary.

TABLE I

ADEQUATE VITAMIN C INTAKE SHOWING MILLIGRAMS EXCRETED IN URINE*

	MILLIGRAMS PER HOUR							
	FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR	TOTAL MG. 4 HOURS	TOTAL MG. 6 HOURS
				(Group 1—Females)				
1.	5.6	4.2	20.3	9.7	12.7	8.8	39.8	61.3
2.	5.5	1.4	7.0	9.0	27.2	19.3	22.9	69.4
3.	3.6	0.0	10.2	9.9	20.7	23.0	23.7	67.4
4.	5.1	14.8	47.0	4.7	5.6	5.1	71.6	82.3
5.	6.4	2.1	5.9	9.9	15.5	7.7	24.3	47.5
6.	4.2	2.5	3.4	7.2	5.9	4.7	17.3	27.9
7.	1.0	1.6	5.6	12.1	23.0	9.4	20.3	52.7
Ave.	4.49	3.80	14.20	8.93	15.80	11.14	31.41	58.36
				(Group 2—Males)				
8.	0.4	6.7	6.1	11.2	9.8	7.3	24.4	41.5
9.	1.6	7.9	3.6	6.2	7.8	7.7	19.3	34.8
10.	1.4	9.4	19.0	7.2	8.2	25.2	37.0	70.4
11.	2.5		18.2	7.2	6.9	13.2	27.9	48.0
12.	1.3	5.5	7.6	6.7	8.1	5.1	21.1	34.3
13.	0.5	0.9	8.5	13.7	7.1	4.8	23.6	35.5
14.	0.5	2.8	9.7	9.8	8.0	7.2	22.8	38.0
15.	0.3	0.4	3.6	16.3	17.7	6.8	20.6	45.1
Ave.	1.07	4.20	9.53	9.79	9.2	9.7	24.59	43.49
				(Averages of Groups 1 and 2)				
	2.56	4.00	11.57	9.41	12.9	10.33	27.54	49.96

*After oral administration of a 200 mg. test of Ascorbic Acid.

The eighteen subjects selected, because their history indicated an inadequate vitamin C diet, excreted, after oral administration of 200 mg. of ascorbic acid, an average of 3.2 mg. for four hours and 5.1 mg. for six hours (Table II).

TABLE II

INADEQUATE VITAMIN C INTAKE SHOWING MILLIGRAMS EXCRETED IN URINE*

	MILLIGRAMS PER HOUR							
	FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR	TOTAL MG. 4 HOURS	TOTAL MG. 6 HOURS
16.	2.1	0.9	0.5	0.4	2.2	0.5	3.9	6.6
17.	0.0	1.0	0.5	0.0	0.3	1.3	1.5	3.1
18.	1.2	0.9	0.0	1.2	0.3	0.9	3.3	4.5
19.	0.5	0.6	1.4	1.0	0.8	1.0	3.5	5.3
20.	0.7	0.4	0.0	1.4	1.1	0.4	2.5	4.0
21.	1.8	0.5	0.4	0.6	1.0	0.5	3.3	4.8
22.	1.0	0.5	0.5	0.5	0.8	0.6	2.5	3.9
23.	0.4	0.6	0.4	0.4	0.6	0.8	1.8	3.2
24.	1.4	0.6	0.8	0.3	0.6	0.6	3.1	4.3
25.	1.0	0.5	0.4	0.7	0.6	0.5	2.6	3.7
26.	0.4	2.5	2.5	0.7	1.0	2.4	6.1	9.5
28.	0.1	0.2	0.5	0.9	0.9	1.2	1.7	3.8
29.	0.2	0.5	0.9	0.8	1.0	1.1	2.4	4.5
30.	0.9	1.0	2.2	0.9	1.9	1.4	5.0	8.3
31.	1.0	1.1	2.8	0.3	1.2	1.7	5.2	8.1
32.	0.5	0.5	1.1	1.1	1.2	0.9	3.2	5.3
33.	0.6	0.7	1.6	0.5	1.0	0.8	3.4	5.2
34.	0.8	0.6	0.6	0.5	0.8	0.9	2.5	4.2
Ave.	0.81	0.76	0.95	0.68	0.96	0.97	3.20	5.13

*After oral administration of 200 mg. of Ascorbic Acid.

The four patients suspected of a vitamin C deficiency were tested with this saturation test (Table III). During the six-hour period, the average excretion was 6.55 mg. After receiving approximately 200 mg. of vitamin C (from fruit

juices) daily for sixteen days, these patients were retested. The average excretion for the 6-hour test period was 118.3 mg. Three of the four patients excreted 116.9, 163.5, and 149 mg. of ascorbic acid, indicating that their tissues were well saturated.

TABLE III

RESPONSE TO DAILY ORAL ADMINISTRATION OF VITAMIN C SHOWING MILLIGRAMS EXCRETED IN URINE*

PATIENT	TEST	MILLIGRAMS PER HOUR						TOTAL MG. 4 HOURS	MG. 6 TOTAL HOURS
		FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR		
29	1st	0.2	0.5	0.9	0.8	1.0	1.1	2.4	4.5
	2nd†	0.3	4.7	28.6	29.7	37.1	16.5	63.0	116.9
30	1st	0.9	1.0	2.2	0.9	1.9	1.4	5.0	8.3
	2nd†	3.0	22.2	31.5	31.1	32.0	43.7	87.8	163.5
31	1st	1.0	1.1	2.8	0.3	1.2	1.7	5.2	8.1
	2nd†	1.1	8.6	31.0	21.0	36.8	50.5	61.7	149.0
32	1st	0.5	0.5	1.1	1.1	1.2	0.9	3.2	5.3
	2nd†	0.2	0.4	7.4	15.5	10.2	10.2	23.5	43.9

*After oral administration (test dose) of 200 mg.

†Second test was made 16 days after the first test; during this interval the patients received approximately 200 mg. of vitamin "C" daily.

TABLE IV

TWENTY-FOUR HOUR URINARY EXCRETION OF VITAMIN C BEFORE AND AFTER ORAL ADMINISTRATION OF ASCORBIC ACID
Showing milligrams per specimen

TIME HOURS	SUBJECT 8		SUBJECT 13		SUBJECT 15	
	BEFORE	AFTER*	BEFORE	AFTER*	BEFORE	AFTER*
1	0.2	1.6	0.2	0.5	0.1	0.3
2	0.4	7.9	0.1	0.9	0.02	0.4
3	†	3.6	†	8.5	0.1	3.6
4	0.5	6.2	0.2	13.7	0.2	16.3
5	2.1	7.8	†	7.1	0.7	17.7
6	1.3	7.7	1.2	4.8	0.4	6.8
8	†	6.5	0.8	3.6	0.2	5.5
10	0.6	3.7	0.5	5.0	1.0	0.8
12	1.4	12.4	0.8	1.8	0.5	0.3
14	0.4	4.2	†	†	†	†
20	†	†	0.0	5.3	†	3.7
22	7.5	4.9	†	†	†	†
24	3.1	6.5	0.9	1.6	0.5	0.6
Total	17.4	73.0	4.7	52.8	3.7	55.9

*After oral administration of 200 mg. of Ascorbic Acid.

†No urine specimen collected.

Another individual with a history of a very inadequate vitamin C diet excreted 3.8 mg. in the 6-hour period. After receiving 50 mg. of ascorbic acid daily for six days, a second test resulted in an excretion of 3.7 mg. After twenty-nine days on this 50 mg. dose, the response to a test dose was 10.3 mg. for the six-hour period. The daily dose was increased to 75 mg. Six days later the response to the test dose produced satisfactory results, with an excretion of 47 mg. in six hours.

Finally, a study was made on three individuals on an adequate vitamin C diet to determine the rate of ascorbic acid excretion during two consecutive twenty-four hour periods (Table IV). During the first twenty-four hour period these men had a normal diet without the test dose. After the last urine specimen

was collected for this first period, these men were given 200 mg. of ascorbic acid, and the urine specimens were collected for the second twenty-four-hour period. The results show that there is a marked difference in the ascorbic acid content of the 24-hour urine specimens before and after a test dose is administered.

SUMMARY

1. Satisfactory results can be obtained with a 200 mg. saturation test.

2. Since the maximum rate of excretion, after oral administration, occurs during the third, fourth, fifth, and six hours, a six-hour saturation test appears to be satisfactory. A four-hour saturation test probably will give satisfactory results. In either case, hourly specimens are not necessary.

3. An adequate vitamin C diet is indicated by a urinary excretion of approximately 20 mg. or more of ascorbic acid in four hours, or approximately 30 mg. or more in six hours. Less than 10 mg. in six hours can be definitely considered inadequate.

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METHODS FOR THE DETERMINATION OF THIOURACIL IN TISSUES AND BODY FLUIDS*

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DURING the past two years the goitrogenic effects of certain thioureas and sulfonamides have been reported by several observers.[1-7] Mackenzie, Mackenzie, and McCollum[1] noted, while studying the effect of sulfaguanidine on the bacterial synthesis of vitamins in rat intestines, that marked enlargement of the thyroid resulted from this drug. Richter and Clisby,[2] when investigating the effects of various unpleasant tasting substances in rats, found that phenyl thiourea induced goiter formation. Kennedy and Purves[3] demonstrated in rats a goitrogenic effect of rapeseed, this effect being ascribed to thiourea.[4] Subsequent studies by Mackenzie and Mackenzie,[5] as well as by Astwood and associates,[6] revealed that a large number of sulfonamides and thioureas caused in rats not only marked thyroid enlargement, but also a decrease in the basal oxygen consumption. Whereas these changes were prevented by hypophysectomy or by the simultaneous administration of thyroxin; diodotyrosine and potassium iodide had no effect. These sulfonamides and thioureas inhibit the production of the thyroid hormone, leading to a fall in the basal metabolism and enlargement of the thyroid gland. Astwood[7] found that 2-thiouracil possessed these properties to a greater degree than any of a large number of substances which he tested. He also reported[8] that three thyrotoxic patients treated with thiourea or thiouracil experienced a fall in the basal oxygen consumption and a remission of their symptoms. Williams and Bissell[9] confirmed these observations and also found that the plasma protein-bound iodine returned to normal in association with thiouracil therapy. An arbitrary dosage of about one gram daily has been used, but it is desirable to ascertain the minimal effective dosage since one case of severe agranulocytosis resulted from the thiouracil treatment.[8] The ultimate solution to this problem must be based upon the response of the patient to varying dosages, but it would be very helpful to know about the rate of absorption from the gastrointestinal tract, the distribution throughout the body, and the rate of excretion of the drug. Several months ago we began an investigation of these points, but since no methods had been reported for the determination of thiouracil in tissues and body fluids, it was first necessary to attack this problem. In this paper we are presenting the methods which we have found to be satisfactory.

The methods chosen are based on Grote's[10] observation that a blue color was formed by substances of a C = S type when treated with a special reagent.

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Grote's reagent was produced by the treatment of sodium nitroferricyanide in sodium bicarbonate solution with hydroxylamine hydrochloride followed by bromine. The reaction is apparently limited to compounds of divalent sulfur linked to a single nonmetallic element; thiouracil (2-thio, 6-oxypyrimidine) has this linkage.

METHODS

A. *Preparation of Grote's Reagent.*—In preparing the reagent used by Grote, 0.5 Gm. of sodium nitroferricyanide is dissolved in 10 c.c. of water in a small beaker at room temperature. To this mixture is added 0.5 Gm. of hydroxylamine hydrochloride and then 1 gram of finely powdered sodium bicarbonate. The beaker is kept covered with a watch glass until the evolution of gas has ceased and then 0.1 c.c. of bromine is added. The cover is replaced until gas no longer evolves. This solution, which is clear mahogany brown, is filtered into a 25 c.c. volumetric flask and then diluted to volume. Before using this solution it is further diluted 1:5.[11]

B. *Determination of a Standard Curve.*—A 10 mg. per cent aqueous solution of thiouracil* is prepared. The pH is adjusted to 8.5 with normal sodium hydroxide, using a glass electrode for the pH determinations. Aliquots of 0.05 to 1 c.c. are diluted with distilled water to 1 c.c. To each tube is added 1 c.c. of Grote's reagent. After standing for fifteen minutes, 4 c.c. of 1 per cent sodium chloride is added to each tube to a total volume of 6 c.c., and the intensity of the color is immediately determined in the Evelyn photoelectric colorimeter, using a 660 filter and No. 6 diaphragm. The tube used as a blank contains 1 c.c. of Grote's reagent and 5 c.c. of one per cent sodium chloride. When the results are plotted on semilogarithmic paper, a straight line is obtained.

C. *Urine Analysis.*—The urine is preserved with chloroform or hydrochloric acid. The pH of the entire specimen is adjusted to form 8.5 to 9 with normal sodium hydroxide. About 10 c.c. of the urine are centrifuged and from 0.25 to 0.5 c.c. of the clear layer is pipetted into a colorimeter tube. Following the addition of 1 c.c. of Grote's reagent, the tube is allowed to stand for fifteen minutes. It is then diluted with 1 per cent sodium chloride to 6 c.c. and read immediately in the colorimeter, as in the case of the standard solutions.

D. *Blood Analysis.*—To 1 c.c. of oxalated blood is added 1 c.c. of distilled water. The tube is shaken and the pH is adjusted to from 8 to 8.5. Then 0.1 c.c. of a 5 per cent solution of trypsin (technical) is added, and the mixture is incubated at 37° C. for twelve hours. A protein filtrate is prepared, using 1 c.c. of the digested blood mixture, 7 c.c. of distilled water, 1 c.c. of 5 per cent copper sulfate, and 1 c.c. of 7 per cent sodium tungstate. The pH of the filtrate is adjusted to from 8.5 to 9. Then 5 c.c. of the filtrate are pipetted into a colorimeter tube, and 1 c.c. of Grote's reagent is added. After fifteen minutes, the amount of color development is estimated in the colorimeter as in the case of the standard solutions. Occasionally a slight cloudiness develops after the addition of Grote's reagent, in which case the solution is filtered through No. 2 Whatman filter paper.

*The thiouracil was supplied by the Lederle Laboratories, Inc., Pearl River, N. Y.

E. *Tissue Analysis.*—To the tissue is added an equal volume of water and about one-half this volume of normal sodium hydroxide. A homogenous suspension is prepared in a Waring Blendor or in a mortar. The mixture is allowed to stand overnight in the icebox, and an aliquot of 1 c.c. is taken for analysis. A protein filtrate is prepared by means of the copper sulfate-sodium tungstate mixture, as in the case of the blood. Following the adjustment of the pH from 8.5 to 9.0, 5 c.c. of the filtrate are pipetted into a colorimeter tube. Then 1 c.c. of Grote's reagent is added, and the color is estimated after fifteen minutes.

COMMENTS

A. *Physical and Chemical Properties of Thiouracil.*—Little or no data on the properties of thiouracil seem to have been published since Wheeler and Liddle [12] reported the synthesis of this compound in 1908. Thiouracil is a white, odorless, bitter powder. It is insoluble in methyl alcohol, ethyl alcohol, carbon tetrachloride, acetone, and mineral acids; it can be partially extracted from an acidified aqueous solution by shaking with ether over a prolonged period of time. A saturated aqueous solution contains about 50 mg. of thiouracil per 100 c.c. at 25° C. Due to its acid properties, it forms very soluble salts on addition of sodium or potassium hydroxide to an aqueous suspension. Solutions of thiouracil are not affected by heating to 80° C. for ten minutes, although they lose 20 per cent of their thiouracil content when kept in a boiling water bath for five minutes; 100 per cent when autoclaved for five minutes at 105° C. under fifteen pounds of pressure.

Dry thiouracil is stable for months at room temperature. It has been found to remain stable for several days when kept in the icebox (5° C.) whether in the form of an aqueous solution, or in urine, blood, or tissue extracts. No destruction occurred when a solution containing 20 mg. per 100 c.c. of thiouracil was exposed to direct sunlight for one hour.

B. *Factors Affecting the Color Reaction.*—The maximal color reaction between thiouracil and Grote's reagent takes place at a pH from 8 to 9. The further the pH is removed from this range, the less intense is the color produced.

Although some color begins to develop as soon as Grote's reagent is added to a solution of thiouracil, the maximal reaction, at room temperature, requires about fifteen minutes. If an interval longer than fifteen minutes is allowed to elapse between the addition of the reagent to the solution of thiouracil and the dilution of this mixture to 6 c.c., the color so produced gradually decreases; after seventy-five minutes, the intensity is only one-half that obtained after fifteen minutes (Fig. 1). On the other hand, the color of the diluted mixture decreases only 3 per cent on standing at room temperature for sixty minutes. The rate of the reaction can be increased by heat; the maximal color at 55° C., 60° C., and 75° C. is obtained in five, four, and two minutes respectively. However, the process of heating may cause some cloudiness which interferes with colorimetric estimations. Light was shown to affect the reaction, since only 40 per cent of the usual color developed when 0.5 c.c. of a 20 mg. per 100 c.c. solution of thiouracil was added to Grote's reagent and permitted to remain in the dark for fifteen minutes.

Undiluted Grote's reagent will remain stable for two weeks or more if kept in the icebox. However, it should be tested with a standard solution of thiouracil at intervals of two to three weeks. The only component of Grote's reagent which we have thus far observed to deteriorate is hydroxylamine hydrochloride. We have found this substance to lose appreciable activity within six weeks, in spite of keeping it stoppered and in the icebox most of the time.

Although a number of factors affect the color intensity, when the determinations are performed in the manner outlined previously, relatively constant results are obtained, making it unnecessary to make frequent estimations on standard solutions.

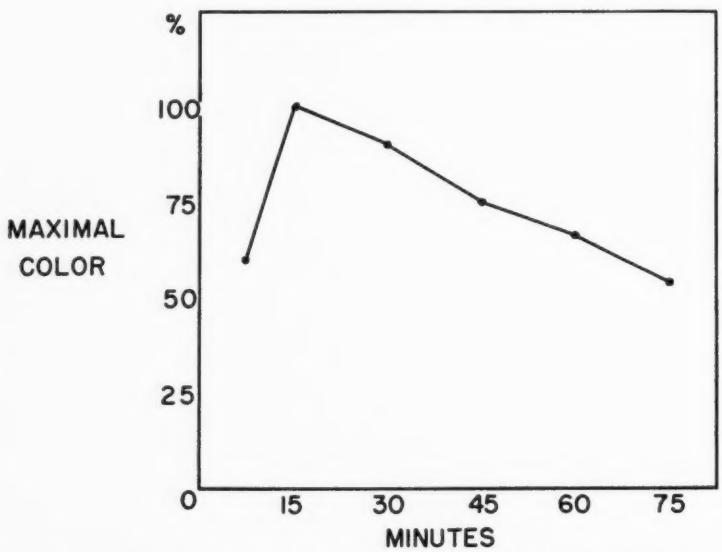


Fig. 1.—The effect of time on the intensity of the color produced by the reaction at room temperature of thiouracil (8 mg. per cent solution) with Grote's reagent.

C. *Analysis of Specimens.*—In all of the analyses conducted we have used as a blank for the colorimeter readings 5 c.c. of distilled water and 1 c.c. of Grote's reagent. This is permissible because all of the specimens to be assayed, except urine, give a clear colorless filtrate, and the quantity of urine used is so small that the yellow color does not interfere with the determination. (1) *Urine.*—Thiouracil is soluble in urine in dilutions of 1:2000, and, therefore, it should be in solution with the dosages ordinarily used therapeutically. When the pH of the urine specimen is adjusted to 8.5, complete solubility is assured. A slight precipitate usually follows alkalinization, but this can be removed by centrifugation. (2) *Blood.*—The analysis of blood proved to be a much more difficult problem than that of urine. It is necessary to obtain a clear filtrate for accurate colorimetric estimations. However, it is found that a large part of thiouracil remains in the precipitate. Zinc sulfate, sodium hydroxide mixture, trichloracetic acid, and tungstic acid all proved to be unsatisfactory as protein precipitants because they render the thiouracil insoluble. Since the concentration of thiouracil in the resulting filtrates is always far below that necessary

to produce a saturated solution, i.e., about 50 mg. per 100 c.c., it seems that thiouracil at an acid pH combines with the precipitated proteins or their breakdown products. This is borne out by the fact that when the proteins of hemolyzed blood were digested with trypsin, recoveries were incomplete when the above precipitants were used. Precipitation with sodium tungstate and copper sulfate, on the other hand, yielded satisfactory recoveries. Thus, the first step in the analysis of blood is to hemolyze it with distilled water. The pH is then adjusted to from 8 to 8.5, since this is the range for maximal activity of trypsin. Incubation is carried out at 37° C. for at least eight hours; shorter intervals lead to incomplete extraction. As the blood becomes more alkaline upon incubation, the protein filtrate is sometimes blue in color due to copper. However, upon reducing the pH to 8.5, the filtrate again becomes colorless. Occasionally, when Grote's reagent is added to the filtrate, a cloudiness develops but this is readily removed by filtration. (3) *Tissues.*—When the quantity of thiouracil is expected to be small, the amount of fluid added should be only enough to permit the formation of a thin emulsion of tissue. Sodium hydroxide is used to extract thiouracil from the tissues; for complete extraction it is advisable to permit the mixture to stand overnight. It is necessary to prepare a protein-free filtrate in order to obtain a colorless solution. Digestion of the tissue proteins with trypsin has been found unnecessary.

TABLE I

RECOVERY OF THIOURACIL ADDED TO THE URINE AND BLOOD

URINE			BLOOD		
ADDED MG./100 C.C.	RECOVERED MG./100 C.C.	PER CENT RECOVERY	ADDED MG./100 C.C.	RECOVERED MG./100 C.C.	PER CENT RECOVERY
0	0	---	0	0	---
2	2	100	0.4	0.32	80
4	3.9	98	1.6	1.4	88
5	5	100	2.4	2.4	100
6	6	100	3.2	3.2	100
8	8	100	4.0	4.0	100
10	10	100	6.0	5.9	98
20	19.8	99	8.0	8.0	100
50	49.2	98	10.0	9.6	96
100	96	96			

The analysis of liver differs slightly from that of the other tissues in that it is advisable to remove the excess fat. A suspension of liver in a weakly alkaline solution is extracted with ether, preferably overnight. The ether is then decanted and the usual procedure is resumed.

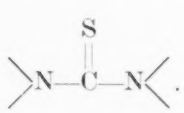
D. *Recoveries.*—In an accompanying paper[13] are presented the results of numerous analyses of the thiouracil content of urine, blood, and tissues of patients. In a number of experiments, thiouracil was added to such materials in quantities sufficient to yield concentrations approximating those obtained in the analyses. Assays of the content of thiouracil in these specimens usually gave values which were from 90 to 100 per cent of the amount added. Very small quantities of thiouracil could be recovered to an extent of not less than around 80 per cent due to analytical error in such low ranges of concentration. The results given in Tables I and II are representative of the ones which we have obtained repeatedly.

TABLE II

RECOVERY OF THIOURACIL ADDED TO TISSUE SUSPENSIONS

ADDED MG./100 C.C.	LIVER		KIDNEY		MUSCLE	
	RECOVERED MG./100 C.C.	PER CENT RECOVERY	RECOVERED MG./100 C.C.	PER CENT RECOVERY	RECOVERED MG./100 C.C.	PER CENT RECOVERY
0	0	---	0	---	0	---
0.4	0.4	100	0.3	75	0.4	100
0.8	0.7	87	0.7	87	0.7	87
1.2	1.2	100	1.3	107	1.2	100
1.6	1.5	94	1.6	100	1.5	94
2.0	2.0	100	2.0	100	2.0	100
4.0	3.8	95	3.8	95	3.7	93
6.0	6.0	100	6.1	101	5.9	98
8.0	7.6	95	7.6	95	7.8	97
10.0	9.6	96	9.8	98	10.0	100
20.0	19.8	99	19.9	99	20.0	100
40.0	38.0	96	39.0	97	38.0	96
60.0	55.0	91	56.0	93	58.0	96
100.0	98.0	98	98.0	98	100.0	100

E. *Evidence for the Identity of Thiouracil in Tissues and Body Fluids.*—To test the specificity of our colorimetric procedure, a great number of possible breakdown products of thiouracil, as well as other types of sulfur compounds, were treated with Grote's reagent. These experiments indicate that an appreciable color with the reagent, under our conditions, is produced only by those possessing the group



Potassium sulfide, sodium sulfite, thiocyanate, and thiosulfate, uracil, urea, creatinine, cysteine, cystine, glutathione, choline, methionine, thiamine, and sulfanilamide were tested in concentrations equivalent to 100 mg. per 100 c.c. of thiouracil. According to Grote's findings,[10] all the compounds in this list which contain divalent sulfur linked to a single nonmetallic element can be expected to yield a color with the reagent. This agrees with our findings that sodium thiocyanate and thiosulfate, in neutral or acid solution, produce a blue color with the reagent; even creatinine will react in this way in acid solution. However, none of the above compounds yielded any color when their solutions were adjusted to a pH from 8.5 to 9 before addition of Grote's reagent, nor did their presence inhibit or enhance the color formation due to 10 mg. per 100 c.c. of thiouracil. Sodium pentothal yielded 10 per cent, and thiobarbituric acid 35 per cent of the color produced by an equivalent amount of thiouracil.

Thiourea, under the above conditions, yields an intense color with Grote's reagent; this color is a pure blue as contrasted with a greenish-blue obtained with thiouracil. Rough absorption spectra of the color of thiouracil and thiourea with the reagent were obtained by determining the absorption of such solutions in the Evelyn photoelectric colorimeter with the filters available in our laboratory; the filters used had their maximum transmission at 420, 440, 520, 540, 565, 620, and 660 mμ. At the same time, similar absorption curves were obtained for urines collected after administration of either thiouracil or thiourea by mouth, with due corrections for the light absorption of the urine and of

Grote's reagent. Fig. 2 shows that there is considerable difference in the absorption curves of the compound formed by Grote's reagent with thiourea and thiouracil, respectively. This was to be expected from the visually apparent difference in color between these compounds referred to earlier.* However, a marked similarity is obvious between the shape of the absorption curve of pure thiouracil or thiourea and of the urines containing the excretion products of either one.† At wave lengths up to 520 mμ, urines treated with Grote's reagent, when corrected for the absorptions of the reagent and the urine, showed some absorption; the same was found for urines devoid of thiouracil or thiourea. Obviously Grote's reagent reacts with some normal urinary constituent to yield a compound which absorbs in this region. Neither the urines nor the reagent showed any appreciable absorption at wave lengths greater than 520 mμ.

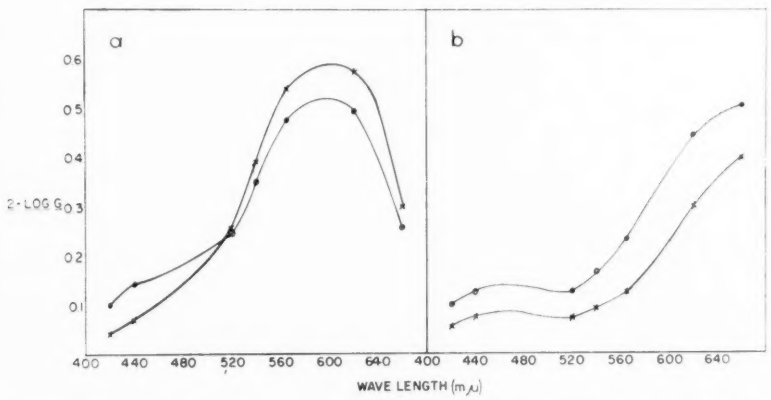


Fig. 2.—*a*, Absorption curves of the reaction product of Grote's reagent with thiourea (x —— x) and with urine containing the excretion product of orally administered thiourea (0 —— 0).

b, Absorption curves of the reaction product of Grote's reagent with thiouracil (x —— x) and with urine containing the excretion product of orally administered thiouracil (0 —— 0).

In several instances, extracts were prepared of liver, heart muscle, and skeletal muscle of guinea pigs which had not been given any thiouracil. These extracts, as well as urines and blood filtrates from patients who had not been treated with thiouracil, never yielded any color when Grote's reagent was added to them.

SUMMARY

1. Methods are described for the estimation of the content of thiouracil in tissues, urine, and blood.

2. The methods depend upon the fact that the $\rangle N—\overset{\overset{S}{\|}}{C}—N\langle$ linkage of thi-

*The validity of the above approximate spectrometric method was established by comparing the absorption curves of Fig. 2 with more complete ones obtained for thiouracil with a Hardy recording spectrophotometer, and for thiourea with a Beckmann spectrophotometer. The two sets of spectra for the same compound were superimposable. Moreover, by extending the spectrum of thiouracil to 700 mμ, it was found to show a maximum at about 680 mμ, thus making it appear very similar to that of thiourea except for a shift of about 90 to 100 mμ towards the red part of the spectrum.

†The differences in the absolute *magnitudes* of absorption, as opposed to the relative absorptions expressed by the *shapes* of the curves, between the pure drugs and their urinary excretion products is due, of course, to differences in the concentration of the chromogen in these respective solutions.

ouracil reacts with sodium aquoferricyanide in alkaline solution giving a greenish color which can be quantitated in a colorimeter.

3. Thiouracil in blood is partially bound to proteins. It can be liberated by tryptic digestion before assaying.

4. Using the methods which we have described, almost complete recovery of added thiouracil can be obtained.

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THE PREPARATION OF A MORE STABLE CONCENTRATED UREASE SOLUTION*

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UREASE solutions prepared by extraction of Jack Bean Meal with alcohol and water are not stable at room temperature and when refrigerated may not be kept safely for more than one month. The alcohol in these extracts may also decrease the stability of the color developed after nesslerization. Water solutions of commercial urease powder are also unstable. Urease powder cannot be conveniently measured uniformly unless weighed; this is a time-consuming procedure.

A simple procedure is proposed for the preparation of concentrated solutions of extracts of Jack Bean Meal and of commercial urease which are stable several weeks at room temperature.

TABLE I

COMPARISON OF FRESHLY PREPARED UREASE, AGED UREASE, AND AGED JACK BEAN MEAL EXTRACT SALT SOLUTIONS IN THE DETERMINATION OF BLOOD UREA NITROGEN

UREASE		JACK BEAN MEAL EXTRACT
FRESH	AGED 17 WK. AT 25° C.	AGED 9 WK. AT 25° C.
MG. PER CENT	MG. PER CENT	MG. PER CENT
11.6	12.4	11.8
10.6	12.3	12.4
10.3	11.3	11.8
10.6	11.5	11.0
16.7	17.7	16.7
10.2	11.1	10.2
11.2	10.8	11.0
14.3	13.9	14.0
13.1	12.8	13.0
13.9	14.2	14.3
12.9	13.2	13.0
15.8	15.6	15.5
12.8	13.2	13.5
18.8	18.8	19.0
18.2	17.9	17.8
13.2	13.0	12.8
9.1	9.2	9.2
20.8	21.2	21.0
21.2	21.4	21.5
17.8	17.5	17.3
13.7	14.0	13.6
57.0	58.0	56.0
17.8	17.7	17.9
12.5	12.7	12.8
17.0	17.3	17.5
73.5	76.0	73.0
19.7	20.2	19.3
16.9	16.2	16.5
11.5	12.0	11.5
18.0 av.	18.4 av.	18.1 av.

*From the Laboratories, McCloskey General Hospital, Temple, Texas.
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PROCEDURE

1. *Preparation of Extract of Jack Bean Meal.*—To 150 c.c. of saturated sodium chloride solution in a 300 c.c. Erlenmeyer flask, add 40 Gm. of Jack Bean Meal, 4 Gm. of permutit, and shake vigorously several minutes. Let the mixture stand twenty-four hours at room temperature. Shake the mixture again a few minutes and then centrifuge. Filter the supernatant solution through ordinary filter paper. Cover filter, as the filtration may require several hours. Keep in a small dropper bottle. Use 1 drop for each 2.5 c.c. of acid tungstate protein free filtrate.

2. *Preparation of Commercial Urease Solution.*—To 50 c.c. of saturated sodium chloride solution, add 2 Gm. of urease (Squibb double strength powder) and shake vigorously a few minutes. Let stand one hour and filter. Cover filter, as this filtration may require a few hours. Keep in bottle with dropper. Use 1 drop of this solution for each 2.5 c.c. of acid tungstate protein-free filtrate.

Ordinary precautions against contamination of the urease solutions should be taken.

The stability of the saturated salt extracts aged from nine to seventeen weeks at room temperature is indicated by the good agreement between old and fresh extracts (Table I). Blank determinations made upon fresh saturated salt extracts and 9 to 17 weeks' old saturated salt extracts used in the preparation of the data in Table I were negative for ammonia nitrogen or ammonia nitrogen-forming elements. The blood urea nitrogen method of Karr[1] was used for the comparison of the extracts. Blood specimens for the data in Table I were taken from hospital patients during routine laboratory examination.

SUMMARY

A procedure is described for the preparation of concentrated extracts of Jack Bean Meal and of commercial urease which are stable several weeks at room temperature.

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